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PRINCIPAL INVESTIGATOR: Olivera J. Finn, Ph.D.

CONTRACTING ORGANIZATION: University of Pittsburgh
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13. ABSTRACT *(Maximum 200 Words)*

Our **Graduate Training Program in Breast Cancer Biology and Therapy** is a multidisciplinary approach focused on an important disease. The specific goals of the Program are: 1) to recruit qualified predoctoral students to breast cancer related research; 2) to educate students in the fundamental principles of breast cancer pathobiology and therapy; 3) to monitor and evaluate the progress of the enrolled students and mentor them in their future career choices; 4) to organize program activities, such as Seminar Series and Journal Clubs, for increased interaction of the student trainees with postdoctoral fellows and faculty interested in breast cancer. We have completed the first year of the training program in which we have closely followed our specific goals. Six students from five disciplines have completed the first year of support. They have all made satisfactory progress illustrated by their publication record. One student has graduated and accepted a research position in the field of breast cancer. They have all actively participated in the Program activities. All continuing students were granted the second year support

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INTRODUCTION

Our **Graduate Training Program in Breast Cancer Biology and Therapy** is a multidisciplinary approach focused on an important disease. The overall philosophy of our training program is to identify qualified graduate students in the existing discipline-based training programs and to interest and educate them in the unsolved problems in breast cancer. By raising their interest and providing them with financial help, we encourage them to apply the tools of their individual disciplines in search of solutions to breast cancer-related problems. Our Training Program intends to expand the existing pool of investigators studying breast cancer. The Program also strives to encourage many of our faculty members, by including them into the list of Program Faculty and by providing support for their graduate students, to focus their research effort at least in part on breast cancer. This is an important programmatic by-product because it takes ongoing interdisciplinary research effort by an array of well-funded investigators and directs it towards the problems of breast cancer.

The specific goals of our Program are:

a) To recruit qualified predoctoral students to breast cancer related research.

b) To educate students in the fundamental principles of breast cancer pathobiology and therapy.

c) To monitor and evaluate the progress of the enrolled students and mentor them in their future career choices.

d) To organize program activities, such as Seminar Series and Journal Clubs, for increased interaction of the student trainees with postdoctoral fellows and faculty interested in breast cancer.

We have completed the first year of the training program in which we have closely followed our specific goals.

BODY (ANNUAL SUMMARY)

Progress in Trainee Recruitment

As soon as we received the award announcement, we initiated a recruitment process. The recruitment process was directed towards highly qualified students who had not yet chosen their research topic or advisor (2nd year students, to be supported in their third and fourth year) or who had recently identified a breast cancer related research project (3rd year students, to be supported in their fourth and fifth year). All training faculty, as well as the community at large, received an electronically transmitted letter informing them of the program and requesting trainee nominations. Applications were submitted also electronically and evaluated by the **Breast Cancer Training Grant Executive Committee**. The current committee members are: Drs. Finn, Lazo, Kuller, Caggiula, Rosenkrantz and Robbins. Applicants were judged based on their performance in the first and second year of graduate school, faculty comments, and a brief written statement of their research interest as related to breast cancer. An effort was made to ensure equitable distribution of fellowships among multiple disciplines and areas of research. The awards were given for a period of two years, pending satisfactory progress in the first year. Six trainees from five different disciplines started their training on the Breast Cancer Training Grant on September 1, 1999.

Progress in Trainee Education and Monitoring of Progress:

Inasmuch as the students supported by this training grant belong to various graduate programs, the formal course work requirements and credits of dissertation research are

determined by their individual programs. **The Training Program in Breast Cancer Biology and Therapy** requires that the students complete an Ethics course offered by the University and attend the weekly conference on Breast Cancer Biology and Therapy organized every Thursday afternoon by Dr. Jean Latimer. Each student is required to present a seminar in this series at least once during the two year period of support under the training grant. The Program Director, Dr. Finn, monitors all seminars campus wide and alerts the trainees to those of special interest to breast cancer. She does that by forwarding the announcements to all the trainees and their mentors, and whenever possible, a few comments regarding the relevance to the training program.

At the end of the first year, all students submitted progress reports. They also formally presented their research in an one day Program Retreat held September 2 (see Appendix). The Director and Co-Director of the Program, the Executive Committee and several training faculty attended the retreat. The second year of funding was approved based on the progress that the students showed in the first year of funding.

Progress in Organizing Program Activities

In addition to several established seminar series that our trainees attend, we have included them as a group in some special events. For example, Dr. Kent Osborne, a well know breast cancer researcher from Baylor College of Medicine was a Visiting Professor in the Department of Medicine. We organized a lunch meeting with him and our trainees at which they discussed their research. We will continue to explore similar opportunities in the coming year.

Research Accomplishments from 9/1/99 until 8/31/2000

Ying Jiang (Dr. Joseph Glorioso III, advisor), Department of Human Genetics, Graduate Program in Molecular Biology. In treating breast cancer and other cancers by gene therapy, a safe and effective vector must be developed. A promising vector, herpes simplex virus (HSV) has several advantages. Its large genome (152 kb) can accommodate large or multiple therapeutic gene(s). In order to design better therapeutic vectors, the biology of the virus should be understood thoroughly first. The topic of this dissertation project is to investigate the promoter region of the viral protein ICP0. A series of deletion mutants of the ICP0 promoter were successfully created. Each of the 50-100 bp deletion constructs removed 2 or 3 potential regulatory elements. The resulting 14 mutagenized promoters as well as wild-type promoter were inserted upstream of a reporter gene, LacZ in the UL41 locus. In transient assays, Vero cells were co-transfected with these mutants and a luciferase expression vector as an internal control. Primary data showed that most of the elements did not exert their effects individually but acted in concert. These mutants are now being used to produce recombinant viruses to investigate the roles of these elements in ICP0 expression in the context of the viral genome during productive and latent infections as well as during reactivation. Elements that are inducible under certain conditions but not essential to primary infection will be useful in vectors that require long term or inducible gene expression and that are less toxic.

No published results are yet available. Ying Jiang's fellowship was extended for the second year.

Michael Forlenza (Dr. Andrew Baum, advisor), Department of Psychiatry, Division of Behavioral Medicine and Oncology. Research has shown that lymphocytes of high-distress patients have reduced DNA repair relative to that of low-distress patients and healthy controls. Furthermore, deficits in repair are associated with an increased risk of cancer.

Using and academic stress model, we hypothesized that students would exhibit lower levels of Nucleotide Excision Repair (NER) during a stressful exam period when compared to a lower stress period. Participants were 19 healthy graduate level students. NER was measured in lymphocytes using the unscheduled DNA synthesis (UDS) assay with slide autoradiography. Contrary to prediction, mean values for NER significantly increased during the higher stress period relative to the lower stress period controlling for background differences in repair. Furthermore, lymphocytes had significantly increased repair of endogenous damage during the higher stress period. Stress appears to directly increase DNA repair. Additionally, stress may increase DNA repair indirectly by increasing damage to DNA. This study has provided preliminary data to form the hypothesis that stress experienced by breast cancer patients may directly affect DNA repair and treatment outcome. The study to directly test this in breast cancer patients is now being designed. This project was presented as a poster at the annual meeting of the American Psychosomatic Society.

Abstracts and manuscripts:

1. Forlenza, M. J., Latimer, J. J. & Baum, A. (2000). Stress and DNA Repair [abstract]. Psychosomatic Medicine, 62 (1), 117.
2. Forlenza, M. J., Latimer, J. J. & Baum, A. (in press). The Effects of Stress on DNA Repair. Psychology and Health: An International Journal.
3. Latimer, J. J., Nazir, T., Forlenza. M., Dimsdale, J., Kanbour-Shakir, A., Flowers, L, & Grant, S. (under review). Unique tissue specific DNA repair capacity exhibited by human breast epithelium. The Breast Journal.
4. Forlenza, M. J., & Baum, A. (in press). Psychosocial Influences on Cancer Progression: Alternative Cellular and Molecular Mechanisms. Current Opinions in Psychiatry.

Michael Forlenza's fellowship was extended for the second year.

Nina Joshi (Dr. Jeanne Latimer and Dr. Steven Grant, co-advisors), Department of Epidemiology, Program in Molecular Toxicology. There is very little direct experimental information about the etiology and development of sporadic breast cancer. The most widely accepted paradigm, supported by epidemiological evidence, is a synthesis of endocrine and somatic mutational models and suggests that estrogen induced cell proliferation increases the likelihood of errors during replication, increasing the endogenous levels of mutations. When these mutations accumulate in cellular oncogenes and tumor suppressor genes they results in the development of cancer. To test this hypothesis, a cellular model needed to be identified that maintained estrogen responsiveness, epithelial architecture and exhibited a low mutational background using the hypoxanthine guanine phosphoribosyl transferase (HPRT) enzymatic selection assay. To date, ten normal breast and four normal endometrial epithelial primary and extended explant cultures have been established. Both the primary Human Mammary Epithelial Cultures and the Human Endometrial Epithelial Cultures maintain normal epithelial architecture identified by the formation of lobules and mammosphere in the breast cultures and luminal and glandular structures in the endometrial cultures. During adaptation to culture conditions, the extended explants become fibroblastic in morphology. When the first endometrium explant (NDE1, passage 19) is placed on matrigel, these cells resume an epithelial morphology resulting in a mixed culture system similar to the primary culture system. Unfortunately, these late passages of NDE1 were not estrogen responsiveness. Additional work is required to determine the kinetics of extinction of estrogen receptors in both the normal breast and endometrial systems. The validation of the

mitogenic/mutagenic theory will provide the scientific evidence necessary to identify estrogen exposure as a biomarker for the identification of women at high risk of developing breast cancer. Furthermore, these results may provide additional molecular targets for breast cancer therapy. There have been no publications yet from this study. Nina Joshi's fellowship was extended for the second year.

Melina Soares (Dr. Olivera Finn, advisor), Department of Molecular Genetics and Biochemistry, Graduate Immunology Program. Epithelial cell mucin (MUC1) is expressed on ductal epithelial surfaces of breast, pancreatic, colon and several other tissues and on tumors arising from these tissues. This PhD project tests questions related to MUC1 specific immunity tested in the MUC1 transgenic mouse model. In the MUC1 Tg. mouse, human MUC1 is expressed under the control of its endogenous promoter and its tissue distribution is similar to that seen in humans. Three different vaccination protocols were tested: 1) MUC1 peptide-pulsed DC, 2) MUC1 peptide+ mGM-CSF and 3) MUC1 peptide+ SBAS2 (an adjuvant from SmithCline Beecham). The adjuvant-based vaccines induced different IgG isotype responses in conventional mice (suggesting the induction of helper cell responses) but only the SBAS2 adjuvant vaccine was able to induce an IgG response in MUC1 transgenic mice. These studies suggest that the MUC1-transgenic mouse does display CD4+ T cell tolerance. In addition, although the peptide-pulsed DC vaccine is capable of inducing a MUC1 specific response in conventional mice, it is not capable of overcoming CD4+ T cell tolerance in MUC1 transgenic mice. Interestingly, the studies suggest that the IFN-gamma induced by the SBAS2 vaccine (which is responsible for the IgG3 antibody switch) is from a non T cell source. Only the peptide-pulsed DC vaccine was capable of causing tumor rejection in conventional and MUC1 transgenic mice. Neither the MUC1-specific nor the tumor rejection responses induced appear to be autoimmune in the MUC1 transgenic mouse.

Manuscripts published and in preparation:

1. Soares, M. M. and O.J. Finn. Different types of MUC1-based vaccination protocols elicited varied immune effector mechanisms in MUC1 transgenic mice that correlated with differences in their tumor rejection potentials. (in preparation)
2. Soares, M. M. and O. J. Finn. MUC1 Mucin as a Target for Immunotherapy of Cancer: MUC1-based immunotherapeutic strategies (2000). In press

Melina Soares' fellowship was extended for another year.

Tracy Chen (Dr. Billy Day, advisor), Program in Molecular Toxicology. Cyanobacteria, also known as blue-green algae, are a public health problem. People drinking cyanobacteria contaminated water by accident or swimming often become ill. Recent research has shown, however, that components from marine algae could protective or used as treatments against human tumors. **Curacin A** is a structurally unique colchicine site microtubule (MT) perturbing agent derived from the lipid fraction of the marine organism *Lyngbya majuscula*, which is a filamentous cyanobacterium. The major purpose of this study is to examine the mechanisms of curacin A's cell toxicity. To measure its potency in growth inhibition and cell cycle changes, antiproliferative and flow cytometric analyses were performed with ten human carcinoma cell lines, such as breast, prostate, and ovarian cancer cell lines. The results showed that all the GI_{50} values in these tumor cell lines were in the nM range. Flow cytometric data show that changes in cell cycle distribution included hypodiploidy (apoptosis) induction, G_1/G_0 phase reduction, and G_2/M accumulation. The data also showed that Raf-1 fragmentation appeared in attached cells and both Raf-1 and Bcl-2 phosphorylation occurred in curacin A-induced detached cells prior to the appearance of

significant hypodiploid populations. In addition, the results from experiments where a partial blockade of the mitogenic pathway showed that inhibition of MEK1-catalyzed phosphorylation of ERK1 enhances the apoptosis-inducing effects of curacin A, thus suggesting potential schemes for combination therapies.

Manuscripts based on this work are in preparation. Tracy Chen was awarded the second year support. She plans to graduate this year.

Vivian Lui (Dr. Leif Huang, advisor), Department of Pharmacology. This project focused on the improvement of gene expression and delivery efficiency of non-viral vectors. In addition, an in vivo model was developed for the assessment of therapeutic effect of therapeutic genes delivered by non-viral vectors. Non-viral approaches based on molecular abrogation of proto-oncogene expression and immuno-cancer gene therapy were studied in this context. To improve gene expression from non-viral vector, an efficient mammalian expression system for expressing a hammerhead ribozyme (U6neuRz) against a human proto-oncogene c-neu was developed. Extremely high level of ribozyme expression (5 x 106 copies/cell) was achieved using the promoter for the U6 snRNA gene. Expression of U6neuRz in human ovarian carcinoma cells (SKOV-3.ip1) resulted in specific down-regulation of the c-neu target at both RNA and protein levels. More importantly, dramatic growth inhibition of SKOV-3.ip1 was observed with transient expression of U6neuRz. We hypothesized that expression of this ribozyme could achieve therapeutic effect in vivo. In a solid tumor model of ovarian carcinoma, systemic delivery of naked DNA carrying the U6neuRz gene resulted in partial regression of tumor. This indicates that the U6neuRz could be used for the treatment of cancers over-expressing c-neu, such as breast and ovarian cancers.

Publications:

1. Lui, V.W.Y., He, Y.K., Falo, L. and Huang, L. Systemic administration of naked DNA encoding IL-12 for the treatment of cervical carcinoma. (Submitted)
2. Lui, V.W.Y., Falo, L. and Huang, L. Systemic production of IL-12 by naked DNA mediated gene transfer: toxicity and attenuation of transgene expression in vivo. (Submitted)
3. Lui, V.W.Y., He, Y.K., Goyal, K., and Huang, L. C-neu (erbB-2/HER-2) hammerhead ribozyme expression from an efficient U6 expression system results in specific downregulation of c-neu in human ovarian carcinoma cells. (submitted)

This student defended her thesis in August 2000 and accepted a Research Associate position in the laboratory of Dr. Xiao-Ming Yin in the Department of Pathology, University of Pittsburgh. She will focus on investigating the molecular defects in apoptosis pathways leading to drug resistance in breast cancer.

UNIVERSITY OF PITTSBURGH GRADUATE PROGRAM IN BREAST CANCER BIOLOGY AND THERAPY
SCIENTIFIC RETREAT AND PROGRESS REVIEW
Saturday, September 2, 2000
8:30 a.m. -12:00 noon
Magee Research Institute

8:30 - 9:00	Breakfast
9:00 - 9:10	Olivera J. Finn, Ph.D., DOD Training grant PI Opening Remarks
9:10 - 9:40	Tracy Chen (Billy Day, Advisor) "Mechanisms of Curacin A-induced Cell Toxicity"
9:40 - 10:10	Melina Soares (Olivera Finn, Advisor) "Breast Tumor Antigen MUC1-Peptide Based Vaccines in Human MUC1-transgenic Mice"
10:10 - 10:30	Coffee break
10:30 - 11:00	Nina Joshi (Steve Grant/Jean Latimer, Advisors) "Mitogenesis and Mutagenesis in the Human Breast and Endometrial Epithelium"
11:00 - 11:30	Ying Jiang (Joe Glorioso, Advisor) "Characterization of the Potential Regulatory Elements in the ICP0 Promoter Region of Herpes Simplex Virus"
11:30 - 12:00	Michael Forlenza (Andrew Baum, Advisor) "Psychological Stress and DNA Repair"

APPENDIX TO THE SUMMARY

1) Key research accomplishments:

- Progress was made on the generation of a Herpes Virus (HSV) vector for use in gene therapy.
- An unexpected observation was made that contrary to the expected result, stress increases rather than decreases DNA repair.
- New explant cultures were established of normal and malignant breast to test the role of estrogen in the etiology of breast cancer.
- Progress was made on the development of MUC1 based breast cancer vaccine.
- A component from marine algae was evaluated in the laboratory as treatment against breast cancer.
- Methods were develop to use non-viral vectors for delivery of therapeutic genes into tumors.

2) Reportable outcomes

- Scientific Retreat and Progress Review held September 2, 2000 (see page 6)

- two published abstracts, one published paper and five manuscripts in press resulted from research of our trainees in the last year.

- no patents or licenses to date

- one trainee received a PhD degree in August 2000

3) An original and two copies of two published abstracts, one published paper and five manuscripts in press are included.

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MUC1 Peptide-based vaccines in Human MUC1 Transgenic Mice.

Melina Soares and Olivera J. Finn. Immunology Program, University of Pittsburgh School of Medicine, Pittsburgh PA 15261.

The tumor antigen MUC1 is expressed in an underglycosylated form on a variety of human adenocarcinomas. A major portion of the extracellular part of MUC1 consists of numerous tandem repeats of a twenty amino acid sequence. Although CTL and antibody responses to MUC1 epitopes present in the tandem repeat have been identified in cancer patients, these responses are not efficient at rejecting MUC1 expressing tumors. We hypothesize that this is due to the lack of T cell help needed to support CTL proliferation and antibody isotype switching. We are testing this hypothesis in the MUC1 transgenic mouse model. We have tested three different vaccines: synthetic 140aa MUC1 peptide + GM-CSF, MUC1-peptide pulsed DC and MUC1 peptide+SB-AS2. We have seen that these three immunization protocols differed in their ability to induce MUC1-specific IgG isotypes. In addition, only the DC and SB-AS2 vaccines induced strong MUC1-specific CTL responses. These vaccines also differed in their ability to cause rejection of MUC1-expressing tumors. We are currently investigating the correlation between tumor rejection and the ability of these immunogens to activate different components of innate and adaptive immunity. (This work is supported by NIH grant 5RO1 CA56103).

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Abstract withdrawn.

The role of $CD4^+$ T cells in facilitating recurrence of tumor resistant to CTL

M.Terabe[1], S.Matsui[1], J.D.Ahlers[1], A.O.Vortmeyer[2], N.Noben-Traut[3], C.Watson[3], D.P.Carbone[4], L.A.Liotta[2], W.E.Paul[3], J.A.Berzofsky[1]. [1]Metabolism Branch, [2]Lab. of Pathology, NCI and [3]Lab. of Immunology, NIAID, NIH, Bethsda MD and [4]Dept. of Med., Vanderbilt Cancer Ctr., Vanderbilt Univ. Sch. of Med., Nashville TN

Using a tumor expressing HIV gp160 as a model viral tumor Ag, we found a growth-regression-recurrence pattern, and used this to investigate mechanisms of immunosurveillance. In vivo depletion of $CD8^+$ cells showed that regression was dependent on $CD8^+$ T cells and escape from CD8 control led to recurrence of the tumor. Depletion of $CD4^+$ cells did not affect initial growth and regression but prevented recurrence of tumors. IL-4R knockout (KO) mice and STAT6 KO mice were protected against recurrence but IL-4 KO mice were not, strongly suggesting a role for IL-13 or other Th2 cytokines besides IL-4 in limiting immunosurveillance. IFN-γ KO mice, deficient in Th1 cells, were more susceptible. Moreover, IFN-γ mRNA was detected in purified $CD8^+$ T cells from CD4-depleted mice at higher levels than in $CD8^+$ cells from CD4 intact mice. Taken together these findings indicate that the quality as well as quantity of $CD8^+$ CTL determines the completeness of immunosurveillance and involves the Th1 cytokine IFN-γ but is negatively regulated by $CD4^+$ T cells producing Th2 cytokines.

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Sialoadhesin-positive macrophages play an essential role in graft-versus-leukemia reactivity.

V. Umansky, S. Müerköster, M. Rocha, P.R. Crocker, V. Schirrmacher, Division of Cellular Immunology, German Cancer Research Center, 69120 Heidelberg, Germany and Department of Biochemistry, University of Dundee, Dundee DD1 4HN, UK

We recently established a graft-versus-leukemia (GvL) murine model in which immune T-cells mediated complete tumor remissions even in advanced cancer. We now show that this adoptive immunotherapy (ADI) is dependent on host macrophages expressing the lymphocyte adhesion molecule sialoadhesin (Sn). Depletion of Kupffer cells in tumor bearing mice during ADI or the treatment of these animals with anti-Sn mAbs led to complete or partial inhibition of the immune-T-cell mediated therapeutic effect. Sn+ host macrophages in livers formed clusters during ADI with donor CD8 T cells. To test for an antigen presentation function of these macrophages, we used as an in vitro model the antigen β-galactosidase for which a dominant MHC class I L^d restricted peptide epitope is known to be recognized by specific CD8 cytotoxic T lymphocytes (CTL). We demonstrate that purified Sn+ macrophages can process exogenous β-galactosidase and stimulate MHC class I peptide restricted CTL responses. Thus, Sn+ macrophages, which are significantly increased in the liver after ADI, may process tumor derived proteins via the MHC class I pathway as well as via the MHC class II pathway, as shown previously. The synergistic interaction between immune CD4 and CD8 T cells during ADI could thus occur in the observed clusters with Sn+ host macrophages.

stress level (all ps>.05). Yet, when chronic controllable background stressors were paired with controllable acute tasks, increases in background stress were associated with a tendency toward increased CV responses to acute tasks (mean effect sizes: SBP z = 1.99, p < .05; DBP z = 1.26, p<.10). Results will be discussed in light of Learned Helplessness and Cost of Coping theories. The possibility that the domains of background stressors are responsible for apparent effects of controllability will also be discussed.

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STRESS AND DNA REPAIR

Michael J. Forlenza, Jean J. Latimer, Andrew Baum, Behavioral Medicine in Oncology, University of Pittsburgh Cancer Institute, Pittsburgh, PA

This study was a prospective, repeated measures, quasi-experimental examination of perceived stress, urinary cortisol, heart rate, blood pressure, and Nucleotide Excision Repair (NER) of damaged DNA in students during both a low stress (time 1) and high stress period (time 2). The low stress period was within two days of returning from spring or summer break, and the high stress period occurred during the week preceding final or board exams. The primary hypothesis was that students would exhibit lower levels of NER in peripheral blood lymphocytes during the stressful exam period than during the low stress period. Participants were 19 healthy first and second-year students recruited from the University of Pittsburgh Medical School (n = 8), Dental School (n = 4), Law School (n = 4), and Pharmacy School (n = 3). NER was measured using the Unscheduled DNA Synthesis assay (UDS) with autoradiography. As expected, the students reported higher levels of perceived stress just before the exams (time 2) than after returning from a summer or spring break, t (13) = -2.53, p < 05. Multivariate tests of significance revealed no differences in heart rate, systolic, or diastolic blood pressure [Wilk's Lambda = .658, F (3, 10) = 1.732, ns] between the low stress and high stress periods. Additionally, there were no differences in urinary cortisol, t (13) = .28, ns. Contrary to our original hypothesis, mean values for NER were *higher* during the high stress period, t (13) = -2.47, p < .05. There were no differences in NER at time 1 or time 2 as a function of gender [low stress, F (1, 17) = .005, ns; high stress, F (1, 12) = .055, ns] or positive family history of cancer [low stress, F (1, 17) = .261, ns; high stress, F (1, 12) = .571, ns]. These results provide evidence linking stress with NER of damaged DNA, an important mechanism in the prevention of accumulated somatic mutations.

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HOPELESSNESS IS ASSOCIATED WITH EMOTIONALLY INDUCED VAGAL ACTIVATION IN CHILDREN WITH ASTHMA: EVIDENCE IN SUPPORT OF A PSYCHOPHYSIOLOGIC MECHANISM

Bruce D. Miller, Beatrice L. Wood, Pediatric Psychiatry, SUNY, Buffalo, NY

Purpose: The **ANS Dysregulation Model of Emotional Influence on Asthma** (Miller, 1987, Miller and Wood, 1995) proposes that depressed, despairing or hopeless emotional states are associated with ANS dysregulation such that a cholinergic bias potentiates vagally mediated airways constriction in asthma. Previous results indicate that a laboratory induced state of sadness/hopelessness is associated with vagal activation and pulmonary instability in children with asthma (Miller and Wood, 1997). However, traditional literature suggests that stress/anxiety (a physiologic state of sympathetic arousal) is associated with asthma. The purpose of this study was to test whether asthmatic children with underlying emotional states of hopelessness, versus anxiety, show increased vagal activation and/or sympathetic arousal during emotional provocation in the laboratory.
Subjects and Methods: 23 children with mild to moderate asthma (aged 8-16, 11 girls) completed the Hopelessness Scale for Children (HSC) and the Multidimensional Anxiety Scale for Children (MASC). They then viewed the movie video "E.T.: the Extraterrestrial" while having their heart rate and respiration continuously monitored. Respiratory Sinus Arrhythmia (RSA) was used as an index of vagal activation and Heart Rate (HR) as an index of sympathetic arousal. Pearson r's were used to assess association among the emotion scale scores, RSA and HR.
Results: Hopelessness was significantly correlated with RSA (r=.47,p<.05) but not with HR (r=-.23,p=.29). Anxiety was not significantly correlated with RSA (r=.18,p>.4) or HR (r=.23,p>.3).
Conclusion: These findings indicate the emotional and physiologic specificity of hopelessness in association with vagal activation. These results support the hopelessness-cholinergic potentiation link in the ANS Dysregulation Model of the Emotional Influence on Asthma.
Acknowledgement: This work was supported in part by NIMH grant K01MH01291.

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FAMILY PATTERNS OF RELATIONSHIP INFLUENCE PSYCHOPHYSIOLOGICAL PROCESSES IN ASTHMA

Beatrice L. Wood, Kendra Klebba, Bruce D. Miller, Pediatric Psychiatry, SUNY, Buffalo, NY

Purpose: The Biobehavioral Family Model (BBFM) posits that specific family patterns of relationship influence psychophysiological processes in physical illness (Wood, 1993). This study tests a revised 1999 BBFM (with attachment added) by investigating the relations among a child=s perceptions of parental conflict and triangulation, parent-child attachment, emotional state, and vagal activation, one mechanism of airway constriction in asthma.
Methods: Twenty-three children with asthma completed the Children=s Perception of Interparental Conflict Scale (CPIC), the Relatedness Questionnaire (R), and the Hopelessness Scale for Children (HSC). Next they engaged in emotionally challenging Family Discussion Tasks (with mother, father and sibling) in order to evoke psychophysiological responses. Heart rate variability was measured throughout to compute amplitude of respiratory sinus arrhythmia (RSA), an index of vagal activation. Student=s t=s and Pearson r=s tested associations.
Results: Children insecurely related to father (IF) reported more parental conflict compared to children securely related to father (SF), (IF: M=14.3 ± 10.2; SF: M=7.9 ±4.9, p < .05), more triangulation in parental conflict (IF: M=5.5 ±3.2; SF: M=1.5 ±1.7, p < .001), more hopelessness (IF: M=4.4 ±2.7; SF: M=1.8 ±1.6, p < .01) and greater RSA during the Family Tasks (IF: M=7.2 ±1.1; SF: M=6.1 ±.88, p < .05). Hopelessness (HSC) and Triangulation (T) both correlated with RSA during the family tasks (HSC: r=.46, p < .05; T: r=.56, p < .05). Insecure relatedness with mother (IM) related only to hopelessness (IM: M=5.0 ±2.8; SM: M=2.7 ±2.3, p < .05).
Discussion: Parental conflict, triangulation, father-child relatedness, child hopelessness and vagal activation are inter-related. These findings support the 1999 BBFM and emphasize the importance of parent-child (especially father) attachment to psychophysiological processes in asthma. Funded by NIMH KO1MH01291.

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THE RELATIONSHIP OF AMBULATORY BLOOD PRESSURE VARIABILITY WITH LABORATORY MEASURES OF HEART RATE VARIABILITY AND BAROREFLEX SENSITIVITY

Richard A. Nelesen, Joel E. Dimsdale, Psychiatry, University of California, San Diego, La Jolla, CA

Relating laboratory data to environmental data is an important aspect of our research. Recently, interest in assessing heart variability (HRV) to assess autonomic functioning has increased. Variability in blood pressure (BPV) relates to autonomic changes, such as the baroreflex (BARO). Decreased BARO sensitivity results in increased BPV. This study looked at the relationship of blood pressure variability, determined from the standard deviation of daytime ambulatory blood pressure (ABP) recordings with BARO sensitivity, and the high frequency power (HF) of HRV during reactivity testing.
We studied 52 subjects with 24 hour ABP monitoring, BARO testing, and HRV reactivity testing. Subjects had ABP measured the day preceding the testing. The average of the BARO response to phenylephrine and amyl nitrite was used. The HF power was measured at baseline and during reactivity. Reactivity HF was determined as the average of the change in HF (delta HF) across 3 stressors. BPV was the SD of the systolic BP.
Using multiple regression, we predicted BPV as a function of BARO, BHF, and delta HF.

On step 1, BARO predicted BPV, (R^2 = .336 p = .006); step 2, Baseline HF did not contribute significant information to prediction off BPV beyond that produced by BARO; step 3, reactivity HF added significant information to BARO in predicting BPV (R^2 = .646, p < .001, R^2 change = .308, p = .001). BPV is predicted independently by BARO and HF reactivity. Together they account of 64% of the variance in BPV.
Thus, we find that two laboratory measures (BARO and HF reactivity) are strong predictors of ABP variability.

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CORTISOL RESPONSE TO PSYCHOSOCIAL STRESS AND AWAKENING IN FORMER PRETERM CHILDREN

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Animal data indicate that early life stress (maternal separation, restraint stress or early handling) can influnece the biological stress response in later life. It is not clear, however, whether also in humans perinatal stress may have an impact on the later psychological and biological stress response. Children, aged 8-14 years who were delivered prematurely (n=16) and sex and age matchend control children who were born full-term (n=16) were investigated. All children were exposed to the "Trier Social Stress Test for Children" (TSST-C) which mainly consists of a free speech and mental arithmetic tasks in front of an audience. Saliva cortisol was measured 35, 25, 15 and 1 minutes before as well as 1, 10, 20, 30 and 40 minutes after the stressor while heart rate was monitored contionously. For assessment of morning cortisol profiles, the children were asked to sample saliva right after awakening and 30, 45 and 60 minutes later on three consecutive days. Additionally, nightly urinary cortisol secretion was assessed.
Analyses of the data indicated that the TSST-C induced significant increases in cortisol and heart rates, however, preterm children showed significantly attenuated cortisol response (area under the curve) in response to the stressor when compared to children born full-term. No differences were observed with respect to morning cortisol levels or urinary cortisol concentrations during the night. Furthermore, personality traits (e.g. anxiety)

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THE EFFECTS OF STRESS ON DNA REPAIR CAPACITY

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Research has shown that lymphocytes of high-distress patients have reduced DNA repair relative to that of low-distress patients and healthy controls. Furthermore, deficits in repair are associated with an increased risk of cancer. Using and academic stress model, we hypothesized that students would exhibit lower levels of Nucleotide Excision Repair (NER) during a stressful exam period when compared to a lower stress period. Participants were 19 healthy graduate level students. NER was measured in lymphocytes using the unscheduled DNA synthesis (UDS) assay with slide autoradiography. Contrary to prediction, mean values for NER significantly *increased* during the higher stress period relative to the lower stress period controlling for background differences in repair. Furthermore, lymphocytes had significantly increased repair of endogenous damage during the higher stress period. Stress appears to directly increase DNA repair. Additionally, stress may increase DNA repair indirectly by increasing damage to DNA.

KEY WORDS: Psychological stress, academic stress, DNA repair, DNA damage, nucleotide excision repair.

Human DNA is constantly exposed to both endogenous (e.g., superoxide and hydroxyl radicals) and exogenous (e.g., ultraviolet radiation, X-rays, chemicals) genotoxic agents (Feigelson, Ross, Yu, Coetzee, Reichardt and Henderson, 1997). Unrepaired damage due to a reduction or loss of DNA repair leads to permanent somatic mutations and an accumulation of mutations within a single cell drives that cell towards malignancy and cancer (Hall and Johnson, 1996; Vogelstein and Kinzler, 1993). There are several DNA repair pathways organized according to the type of damage repaired or the mechanism of repair (Bohr, 1995; Friedberg, Walker and Siede, 1995). For example, oxidative damage is repaired by the Base Excision Repair (BER) pathway, which removes a single damaged base (Yu, Chen, Ford, Brackley and Glickman, 1999). Lesions induced by UV radiation are repaired by the Nucleotide Excision Repair (NER) pathway, which removes a long 22–30 bp patch of DNA (Yu *et al.*, 1999). BER, NER, and Double Strand Break (DSB) repair pathways repair lesions induced by ionizing radiation. (Yu *et al.*, 1999)

People who inherit DNA repair deficiency syndromes such as xeroderma pigmentosum (XP), Bloom's syndrome, ataxia telengiectasia (AT), or hereditary nonpolyposis colorectal cancer (HNPCC) are at increased risk for developing cancer (Setlow, 1978; Bonn, 1998).

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For instance, people born with XP are prone to skin cancer because they have a mutation in an NER gene that prevents them from repairing damage caused by exposure to UV light (Bootsma *et al.*, 1995; Bonn, 1998). People with HNPCC have a mutation in a gene encoding for mismatch repair, a system involved in correcting replication errors (Jiricny, 1994).

The connection between DNA repair and cancer is not limited to people with inherited syndromes. DNA repair deficits (NER) in blood (PBL's) have been linked to sporadic cancers such as basal cell carcinoma (Wei, Matanoski, Farmer, Hedayati and Grossman, 1994), breast cancer (Kovacs, Stucki, Weber and Muller, 1986), lung cancer (Wei, Cheng, Hong and Spitz, 1996) and head and neck cancer (Cheng, Eicher, Guo, Hong, Spitz and Wei, 1998).

In addition, research has shown that increasing age, independent of antioxidant status, is associated with a decrease in oxidative repair and an increase in mutation in lymphocytes (Bootsma *et al.*, 1995). Aging was also associated with a decrease in the ability to repair UV damage and an increase in DNA mutability in both cultured skin and blood cells (Barnett and King, 1995). Finally, an age-dependent decline in DNA repair has been linked with increases in accumulated mutations in lymphocytes (Moriwaki, Ray, Tarone, Kraemer and Grossman, 1996). However, little work has focused on examining modifiable factors that contribute to the normal variation in DNA repair. Understanding these factors is important for understanding the complex process of carcinogenesis.

There is some evidence that stress can affect DNA repair. In a sample of nonpsychotic psychiatric inpatients, high-distress patients had reduced repair in lymphocytes (i.e., decreased nucleoid sedimentation rates) two hours and five hours after X-irradiation relative to low-distress patients and matched controls. In addition, repair rates in the psychiatric patients were reduced 5 hours post-irradiation compared with healthy controls (Kiecolt-Glaser, Stephens, Lipetz, Speicher and Glaser, 1985). This study provided the first evidence that stress altered DNA repair capacity in humans. If stress suppresses aspects of DNA repair, there may be important consequences for several aspects of cancer prevention and control.

Stress has also been shown to inhibit the repair of carcinogen-induced DNA damage in rats (Glaser, Thorn, Tarr, Kiecolt-Glaser and D'Ambrosio, 1985). Rats were given 50 parts per million (ppm) of dimethylnitrosamine (DMN; a carcinogen) in their drinking water for 16 days and were randomly assigned to a stress or no stress condition. The stress condition consisted of four 24-hr periods of rotational stress in their home cage. After 16 days, the stressed animals had significantly less spleenic methytransferase activity (an important DNA repair enzyme) than the nonstressed animals that had the same level of carcinogen exposure. This suggests that stress may moderate the effect of genotoxic exposures via alteration of DNA repair mechanisms.

The primary objective of this research was to evaluate the relationship between stress and repair of exogenously damaged DNA. Academic examinations are commonplace stressors that have been studied extensively. They are reliably associated with alterations in erythron variables (e.g., hematocrit, hemoglobin, mean corpuscular volume) (Maes, *et al.*, 1998), wound healing (Marucha, Kiecolt-Glaser and Favagehi, 1998), total serum protein (Van Hunsel *et al.*, 1998), serum immunoglobulins, complement, and acute phase proteins (Maes *et al.*, 1997), reactivation of latent Epstein-Barr Virus (Glaser, Pearl, Kiecolt-Glaser and Malarkey, 1994), and inhibition of radiation-induced apoptosis (Tomei, Kiecolt-Glaser, Kennedy and Glaser, 1990). These data suggest that an examination stress model may be a productive way of studying the effects of stress on DNA repair.

METHODS

This study was a prospective examination of perceived stress and DNA repair in 19 students during both a low stress (time 1) and higher stress period (time 2). The low stress period was scheduled within 2 days of returning from spring or summer break, and the higher stress period occurred during the week preceding final or board exams. The primary hypothesis, derived from Kiecolt-Glaser *et al.* (1985), was that students would exhibit lower levels of DNA repair in peripheral blood lymphocytes during the stressful exam period than during the (relatively) low stress period. This study was conducted after review and approval by the University of Pittsburgh Institutional Review Board.

Participants

Participants were healthy first and second-year students recruited from the University of Pittsburgh Medical School ($n = 8$), Dental School ($n = 4$), Law School ($n = 4$), and School of Pharmacy ($n = 3$). Exclusion criteria included smoking, personal history of cancer, and personal history of major psychiatric disorder. Subjects were paid $10 at the end of each session.

Procedure

Upon arrival at the testing site, the study and procedures were explained to the participants and informed consent was obtained. After the subjects completed background and perceived stress questionnaires, 30 ml of blood were drawn into three 10 ml green top tubes (Vacutainer Brand) by a trained phlebotomist or medical assistant. Subjects were then given written information about the time and date of their second appointment and were paid. Procedures for both the low and higher stress periods were similar.

Measures

Basic demographic information and family history of cancer were measured as background variables. In order to evaluate whether the higher stress period was perceived as more stressful, stress appraisal was measured on the Perceived Stress Scale (PSS), a 14-item self-report measure of the extent to which respondents feel their lives are unpredictable, uncontrollable, and overloaded (Cohen, 1986). The PSS demonstrates good internal consistency ($\alpha = .85$) (Cohen, Kamarck and Mermelstein, 1983).

NER was measured in peripheral blood lymphocytes using the unscheduled DNA synthesis (UDS) assay with autoradiography (Cleaver and Thomas, 1981). UDS is a robust functional assay that measures the amount of [^{3}-H]-thymidine incorporated into DNA after an exogenous dose of damaging UV-C light *in vitro*. This permits the quantitative measurement of overall genomic repair (both transcribed and untranscribed genes). The advantage of using a functional assay for NER is that the assay can examine the coordinated functioning of all of the gene products (approximately 30, 11 of which have been cloned) that operate in this repair pathway (Latimer, Hultner, Cleaver and Pedersen, 1996). Additionally, the autoradiographic procedure for UDS, as opposed to the scintillation counting method, allows for a clear-cut elimination through visual inspection of cells incorporating [^{3}-H]-thymidine that are in S-phase.

Blood Preparation

Blood samples were obtained from the participants via standard phlebotomy procedures. Peripheral blood lymphocytes (PBL) were isolated by Ficoll-Hypaque Gradient Centrifugation (Coligan, Kruisbeek, Margulies, Shevach and Strober, 1995). After the final wash, pellets were resuspended in media containing RPMI (Beckman TJ-6) supplemented with 10% autologous serum and 1% pen/strep (Gibco). Autologous serum was used because it contains relevant stress hormones (e.g., cortisol) or biological response modifiers characteristic of the person from whom the sample was obtained (Larcom and Smith, 1988; Larcom, Morris and Smith, 1990). These factors would not be present in samples incubated with fetal bovine sera. Aliquots were placed into culture on chamber slides coated with a diluted form of Matrigel (Collaborative Research, Inc.). This allowed the attachment of the PBLs to the slide (Latimer *et al.*, 1996; Latimer *et al.*, submitted), enabling autoradiographic analysis. Three chamber slides were prepared for each subject.

Assay Procedures

After 3 days in culture with autologous serum and without passaging, the PBL's were assayed for NER. One side of each slide was irradiated with UV-C light (254 nm at a mean fluence of $1.2\,J/m^{-2}s^{-1}\times12$ seconds for a total of $14\,J/m^2$) using a machine specifically built to deliver a reproducible dose of UV radiation (Steier and Cleaver, 1969). The unirradiated side of the slide served as an untreated control and reflected the rate of NER prior to exogenous damage. Immediately following irradiation, samples were cultured in labeling medium containing 10μCi ml [^{3}H]-methyl-thymidine (80Ci/mmol) (NEN Dupont) and allowed to repair for 2 hours at 37°C. The kinetics of UV repair is such that 2 hours of incubation are enough time to repair induced 6–4 photoproducts but not pyrimidine dimers (Latimer *et al.*, submitted). After 2 hours, the labeling medium was removed and replaced with a chasing medium containing non-radioactive thymidine (Sigma) allowing any residual radioactive thymidine to be removed from the intracellular nucleotide pools. The slides were then immersed in photographic emulsion (Kodak type NTB2), and allowed to develop for 11 to 15 days in complete darkness (Cleaver and Thomas, 1981). Tester slides with control cells (foreskin fibroblasts and MDA MB231) were used to assess the optimal time of exposure.

In addition to the peripheral blood lymphocytes, human foreskin fibroblasts were plated, irradiated, and labeled as described for the lymphocyte samples for each experiment. These cells have been documented to have the highest level of NER in mammals and have traditionally served as positive internal controls for the UDS assay (Latimer *et al*, 1996; Latimer *et al.*, submitted). Normal foreskin fibroblasts have also traditionally been used as a standard in the clinical diagnosis of the classic NER disorder xeroderma pigmentosum, a recessively inherited deficiency in the repair of UV damage.

Determination of NER of exogenously damaged DNA. Following development of the slides, the nuclei were stained with Giemsa and the number of radiolabled grains over the nuclei of 88 non-S-phase cells per chamber was counted at 1000X magnification under oil-immersion. Local background counts for each microscopic field were subtracted from the grain counts of each nucleus as a correction. The average number of grains per nucleus for both the irradiated and unirradiated sides of the slide was then calculated. The final values for the mean number of grains per nucleus for each slide were calculated by

subtracting the corrected unirradiated mean grains per nucleus from the corrected irradiated means.

(corrected avg.#grains/ nucleus irradiated) –
(corrected avg. # grains/ nucleus unirradiated)
=average # of grains/ nucleus for each slide

Mean grain counts for each subject were determined by averaging the mean counts for each of the three slides. This represents the corrected average number of grains over the nuclei of at least 276 cells per subject. Results are expressed as a percentage of irradiated foreskin fibroblast repair, the positive standard of comparison for each experiment.

[(Average # grains/ nucleus lymphocytes) /(average # grains/ nucleus FF)] × 100
Normalizing the average number of grains per nucleus in lymphocytes to that of foreskin fibroblast, run in the same experiment, enables inter-assay comparisons and controls for inter-assay variation.

Determinations of NER of Endogenously Damaged DNA. Levels of repair of endogenous damage were calculated using the average number of grains per nucleus from the unirradiated side of the chamber slide. Mean grain counts for each subject were determined by averaging the mean counts for each of the three slides. Results are expressed as a percentage of irradiated foreskin fibroblast repair, the positive standard of comparison for each experiment. As previously stated, normalizing the average number of grains per nucleus in lymphocytes to that of foreskin fibroblasts, run in the same experiment, enables inter-assay comparisons and controls for inter-assay variation.

RESULTS

Demographics. Nineteen subjects completed the protocol at time 1. The mean age of the sample was 25.3 years (range = 19–35). The sample was 68% female ($n = 13$) and 84% of participants ($n = 16$) described themselves as Caucasian. Eighty-nine percent ($n = 17$) were single and never married. This sample consisted of students in a professional academic training program; all of them had completed at least 16 years of schooling. Sixty-three percent ($n = 12$) reported at least one family member with cancer. Demographic data are summarized in Table 1.

Fourteen of the nineteen subjects returned to provide data for time 2. Values for the demographic variables, PSS scores, and NER at time 1 did not differ significantly between those who completed both time points and those who did not complete the second assessment. The demographic characteristics of the sample did not change. All subsequent analyses were carried out using data from only those subjects who completed both assessments.

For the manipulation check, paired *t*-tests were performed on the measures of perceived stress. As expected, the students reported significantly higher levels of perceived stress just prior to exams (time 2) than after returning from a semester or spring break, $t(13) = -2.53$, $p < 05$, two-tailed.

DNA Repair. In order to test the primary hypothesis that DNA repair would be reduced during the examination period, mean values for NER were compared, again using a paired *t*-test. Contrary to our original hypothesis, mean values for NER during the examination period ($M = 9.58\%$, $SD = .79$) were significantly *greater* than mean values for NER during the low

Table 1 Sample demographic characteristics

Variable	*Mean (SD)*	*Frequency*	*%*
Age in years	25.3 (4.34)		
Gender			
Male		N = 6	31.6
Female		N = 13	68.4
Ethnicity			
African-American		N = 1	5.3
Asian		N = 1	5.3
Caucasian		N = 16	84.2
Other		N = 1	5.3
Marital Status			
Single		N = 17	89.5
Married		N = 1	5.3
Divorced		N = 1	5.3
Family History of Cancer			
Yes		N = 12	63.2
No		N = 7	36.8

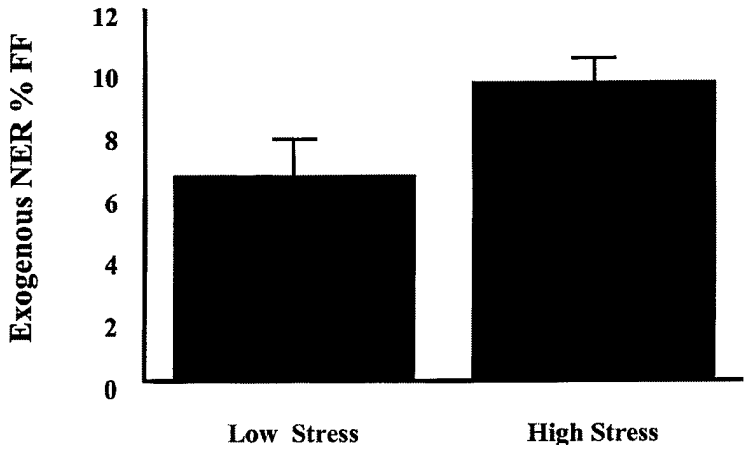

Figure 1 Repair of exogenously damaged DNA by stress period.
Note: Mean values for NER are significantly higher during the high stress period, $t(13) = -2.75, p < .05$.

stress period ($M = 6.23\%$, $SD = 1.31$), $t(13) = -2.47, p < .05$ (see Figure 1). There were no differences in DNA repair at time 1 or time 2 as a function of gender or family history of cancer.

To examine the possibility that increased NER might be a function of endogenous DNA damage, secondary analyses with the NER values of the lymphocytes from the unirradiated side of the chamber slide were conducted. This allowed us to examine preirradiated levels of NER during the exam and non-exam periods. While this value is typically removed from the calculation of NER as a background control, it has previously been viewed as an index of DNA repair that may reflect pre-existing levels of DNA damage (Fischman, Pero and Kelly, 1996). The levels of NER during the exam period ($M = 67.78\%$ $SD = 25.19$) were almost twice that for the non-exam period ($M = 36.63\%$ $SD = 17.74$), $t(13) = -4.99$, $p < .001$, suggesting that stress may be damaging to DNA in a way that can be remediated by the NER pathway (see Figure 2).

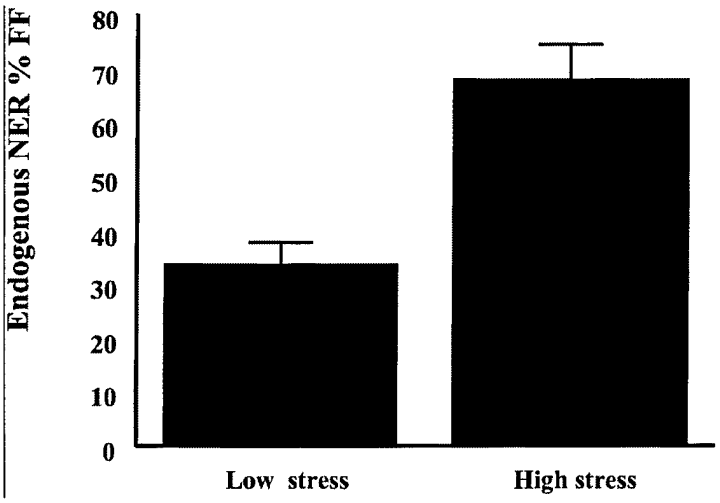

Figure 2 Repair of endogenously damaged DNA by stress period.
Note: Mean values for NER are significantly higher during the high stress period, $t(13) = 4.99, p < .001$.

DISCUSSION

This study tested the hypothesis that stress associated with academic examinations can inhibit the repair of exogenously damaged DNA. This hypothesis was examined by evaluating the NER capacity of peripheral blood lymphocytes from healthy, nonsmoking, graduate students during both a low and high stress period. Contrary to prediction, DNA repair increased significantly during the examination period and was independent of age, gender, and family history of cancer. Thus, stress may account for some of the non-age related variation in NER that has been reported previously (Setlow, 1983; Wei *et al.*, 1994; Grossman, 1997).

Additionally, stress may increase DNA repair indirectly by increasing damage to DNA. In other words, stress may have direct or indirect genotoxic effects that would require adaptive increases in DNA repair to keep the rate of mutation the same. We examined this possibility in secondary analyses with the NER values of the lymphocytes from the unirradiated side of the chamber slide. As noted previously, increased levels of NER prior to exogenously induced damage may reflect increases in endogenous DNA damage (Fischman, Pero and Kelly, 1996). The observed increase in unirradiated levels of NER during the high stress period lends support to the hypothesis that the higher levels of DNA repair observed during the high stress examination period were due at least in part, to greater DNA damage (see Figure 3).

These results are different from those reported by Kiecolt-Glaser *et al.* (1985) and the extensive dissimilarities in both the samples and the methods of these studies make comparison somewhat problematic. However, these differences may account for some discrepancies. Severity of stress is likely to be important, and the present study measured modest, transient stress in a sample of young, healthy, nonsmoking, community-dwelling students. Kiecolt-Glaser *et al.* (1985) measured more severe distress in psychiatric inpatients. Another important difference concerns the timing of the putative stressor. Exam related stress could be considered an acute or episodic event of a relatively fixed duration. In contrast, mental

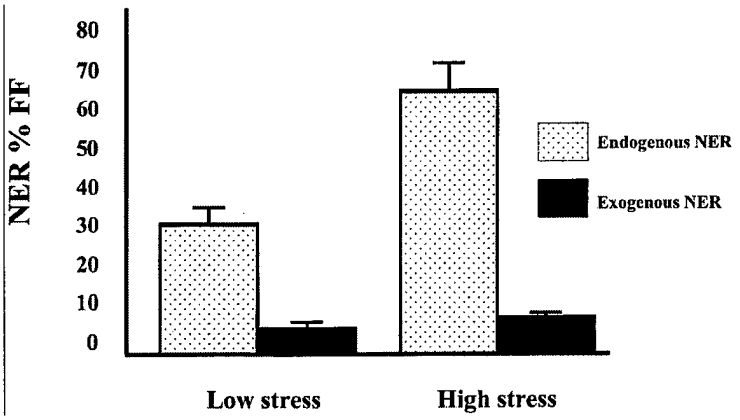

Figure 3 Repair of exogenously and endogenously damaged DNA by stress period.
Note: Mean values for NER of exogenously damaged DNA are significantly higher during the high stress period, $t(13) = -2.75, p < .05$.
Mean values for NER of endogenously damaged DNA are significantly higher during the high stress period, $t(13) = 4.99, p < .001$.

illness severe enough to warrant hospitalization is typically more chronic in nature. The differential effects of acute and chronic stress exposure on DNA repair are unknown.

Additionally, each study examined different repair systems. The present study looked at the NER of UV damaged DNA. UV light creates very specific types of lesions in DNA (i.e. 6–4 photoproducts and pyrimidine dimers). These lesions are remediated specifically though the NER pathway. In contrast, X-ray damage induces a much wider variety and severity of lesions (e.g., single and double strand breaks, oxidative damage) requiring the involvement of many different repair systems including NER. It is not clear how the mechanisms of UV and X-ray damage repair relate to each other.

Another difference concerns preparation of the cells prior to irradiation. We cultured our cells in media supplemented with autologous serum, not fetal bovine serum (FBS). Larcom, Morris and Smith (1990) report that autologous serum always yields higher values than FBS for UDS reasoning that the serum contains biological response modifiers that could affect cellular processes related to repair. This may also partially account for our higher values and the persistence of the stress effects across three days of culture.

Finally, our study allowed only two hours for repair of the DNA before removing the radioactive label. Kiecolt- Glaser *et al.* (1985) measured repair at 0, 2, and 5 hours. When compared with healthy blood donors, differences were not apparent until the repair process had gone on for 5 hours, with no difference in repair observed at two hours. The many differences in stress -related and DNA repair-related variables suggest that future studies will need to pay closer attention to the various methodological details in measurement of DNA damage and repair.

Our results also differ from those of Glaser *et al.* (1985). This is not surprising as there are differences in the DNA repair systems of rats and humans (Cleaver, Speakman and Volpe, 1995). For example, UDS in human cells is 5–10 fold higher than in rodent cells and excises more pyrimidine dimers (Layer and Cleaver, 1997). Additionally, rodents appear to repair damage in genomic regions that are actively transcribed while humans repair both transcribed and untranscribed regions (layer and Cleaver, 1997). Finally, Glaser *et al.* (1985) did not measure NER, but the expression of spleenic methytransferase activity which functions as part of the Base Excision Repair (BER) pathway.

Despite the small size of our study sample, our findings are statistically significant. The within-subjects design provides more power than a between-subjects design with a similar sample size and is unprecedented in the DNA repair literature. Additionally, small sample size (and the resultant low power) often contributes to the inability to find significant differences that truly exist. Our significant result argues against this possibility. However, we do acknowledge that our small sample size is a limitation that argues for caution when generalizing these results. A larger more representative sample would increase variance and could yield different results.

A selection bias may also exist in our volunteer sample. However, the bias seems to have favored those students that were least stressed during exams. That is, the most distressed students did not participate because they did not want their blood drawn immediately prior to their exams. This made detection of a significant difference more difficult, not less. Despite this fact, the difference in repair was still significant.

A third variable other than stress may be responsible for the observed increase in repair. Changes in diet, exercise, sleep or some other unidentified variable may influence DNA repair. The lack of a control group does not allow us to evaluate these alternative hypotheses.

We have suggested that studying the relationship between stress and DNA repair could help explain variation in DNA repair capacity and the subsequent long-term cancer risk. This in turn may offer insight into discrepancies in the literature looking at stress and cancer. Previous research examining the effects of stress on cancer has often led to inconsistent and inconclusive results (Cassileth, 1996; Fox, 1995; Hilakivi-Clarke, Rowland, Clarke and Lippman, 1994; Garssen and Goodkin, 1999; McGee, Williams and Elwood, 1996; Spiegel and Kato, 1996). Methodological problems such as recall and reporting biases, inadequate timing of stress measurements and failure to account for strong biological (e.g., stage of tumor) or behavioral (e.g., smoking history) factors, have made drawing firm conclusions impossible. More importantly, efforts to link stress with the etiology of cancer have failed to identify the intrinsic genetic mechanisms that are affected by stress and that influence neoplastic growth. Research in this area is strengthened by focusing on potential biological mechanisms such as increases in DNA damage, alterations in the amount or rate of DNA repair, inhibition of apoptosis, increases in somatic mutations, and failures of immune surveillance.

Contrary to our hypothesis, we found that NER of exogenously damaged DNA was increased during a period of high stress in young healthy students. Furthermore, we found increases in levels of NER prior to irradiation suggesting as one possibility that stress may be causing an increase in endogenous DNA damage. There is a small literature supporting the relationship between stress and DNA damage in animals. Psychological stress has been shown to damage DNA at the molecular (Adachi, Kawamura and Takemoto, 1993) and chromosomal levels in rats (Fischman, 1989; Fischman, Pero and Kelly, 1996), but there are no such studies in humans. These findings suggest that damage studies directly measuring DNA adducts or excreted DNA damage products should be a priority in research on stress and carcinogenesis.

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Systemic Administration of Naked DNA Encoding IL-12 for the Treatment of Cervical Carcinoma

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Abstract:

IL-12 is one of the most effective and promising cytokines for cancer therapy. Its therapeutic effects have been demonstrated in a variety of tumors in animal models when it is administrated locally or systemically. We describe here a systemic delivery of naked murine IL-12 (mIL-12) gene *in vivo*. A dose-dependent systemic production of mIL-12, with a serum level up to approximately 20 μg/ml of mIL-12, was observed at 24 h after systemic gene delivery. The apparent half-life in the circulation was about 5 h. The result of a bioactivity assay (in vitro IFN-γ release) indicated that the gene product in mice was as active as the purified recombinant murine IL-12 protein (rmIL-12). The circulating mIL-12 activated natural killer cells and stimulated IFN-γ production. A single administration of mIL-12 gene resulted in prominent regression of established subcutaneous tumor in a cervical carcinoma model (TC-1) in C57BL/6J mice. The antitumor effect of the single gene dose was comparable to a repeated intraperitoneal administration of rmIL-12 (0.5 μg/day for consecutive 5 days). This systemic gene delivery is simple, economical and highly efficient for the production of large amount of cytokine in vivo. With this gene delivery method, we have demonstrated the therapeutic potential of IL-12 for the treatment of cervical carcinoma and the usefulness of the systemic gene delivery for assessing the therapeutic effect of a candidate gene.

Overview Summary:

By employing the hydrodynamics-based liver gene delivery of naked mIL-12 gene, we have observed the production of high-level secretable murine IL-12 (mIL-12) in mice. The level of mIL-12 in serum was dependent on gene dose and declined with time. The gene product was functionally active in vitro, with bioactivity comparable to that of purified recombinant mIL-12 protein for the treatment of cervical cancer in an animal model.

Introduction:

Functionally active interleukin-12 (IL-12) is a heterodimer (p70) composing of two subunits (p35 and p40) encoded by two different genes. The two subunits are covalently linked by a disulfide bond to form an active cytokine molecule. The homodimer of p40 is antagonistic to the activity of IL-12 p70 (Gillessen *et al.*, 1995). IL-12 has been shown to have many different biological activities, including the induction of interferon gamma (IFN-γ) production from natural killer cells (NK) and T cells, induction of Type 1 T helper (Th1) cell maturation (Manetti *et al.*, 1993), the ability of enhancing NK and CTL activities, regulation of the production of many other cytokines, stimulation of hematopoietic stem cell proliferation (Jacobsen *et al.*, 1993; Brunda, 1994; Wolf *et al.*, 1994). Owing to its many beneficial biological activities, IL-12 has been evaluated as a therapeutic agent for the treatment of cancer, infectious disease and allergic disorder.

IL-12 is one of the most potent antitumor cytokines in a number of tumor models, including cancers of breast, kidney, ovarian, lung and brain, as well as fibrosarcoma and melanoma (Dias *et al.*, 1998; Sumimoto *et al.*, 1998). For most of the studies, local or systemic administration (by intraperitoneal route) of recombinant IL-12 protein or IL-12 gene was used. The systemic immuno-stimulating effects of this cytokine make it attractive for the treatment of metastases or distant tumors (Mu *et al.*, 1995). Moreover, IL-12 confers

protective activity in vaccination studies (Rodolfo *et al.*, 1996; Sun *et al.*, 1998).

Cervical cancer is the second most common cancer in women and it causes about half a million deaths each year worldwide. Etiological studies showed that infection with human papillomavirus (HPV), especially HPV type 16, greatly increases the risk of developing cervical carcinoma (Josefsson *et al.*, 2000). Vaccination and antisense approaches targeting HPV oncoproteins have shown some success in some experimental models. Vaccination of IL-12 with HPV E6 DNA resulted in enhanced antitumor response (Tan *et al.*, 1999). Our earlier observation that local administration of liposome-IL-12 DNA complexes could result in partial antitumor response (which was synergized by E7 antisense in cervical cancer treatment) demonstrated that IL-12 cytokine therapy might be effective for the treatment of cervical cancer (He *et al.*, 1998).

Owing to its promising therapeutic effects, many different approaches were employed for the delivery of IL-12, including purified recombinant protein and *ex vivo* delivery of engineered fibroblast (Kang *et al.*, 1999). Viral vectors for IL-12 gene delivery have been explored, which include adenovirus construct (Siders *et al.*, 1998), retroviral vector (Tahara *et al.*, 1994), herpes simplex virus (Parker *et al.*, 2000), canarypox virus vector (Puisieux *et al.*, 1998) and adeno-associated virus (Paul *et al.*, 2000). For non-viral methods, particle bombardment of naked IL-12 DNA coated on gold beads (i.e., gene gun) has resulted in the eradication of different types of established primary and metastatic tumors

(Rakhmilevich *et al.*, 1997). Moreover, intradermal injection of plasmid DNA (Tan *et al.*, 1996) and intratumor injection of liposome-mIL-12 DNA complex have also resulted in some antitumor effects (He *et al.*, 1998). For the administration of a cytokine gene, non-viral gene delivery may be more advantageous as it induces minimal immune response compared to viral vectors. However, at this moment, gene delivery efficiency of most of the non-viral methods is still lower than those of viral methods.

In this study, we took advantage of an efficient systemic non-viral gene delivery method, the hydrodynamics-based liver gene delivery (Liu *et al.*, 1999), for *in vivo* mIL-12 gene transfer. Functional comparison of the gene product and the purified recombinant mouse IL-12 protein (rmIL-12) was carried out. We also investigated the antitumor effect of the systemic IL-12 gene transfer in a subcutaneous cervical carcinoma model in comparison with rmIL-12. Our results demonstrated that mIL-12 gene therapy was effective for the treatment of cervical carcinoma in a HPV-16 DNA positive tumor model.

Materials and Methods:

Plasmid Constructs

Plasmids pNGVL3-mIL12 and pNGVL-1 were kind gifts from the National Gene Vector Laboratory at the University of Michigan, MI. The pNGVL3-mIL12 plasmid carries the murine p35, an internal ribosome entry site and the p40 gene driven by a single CMV promoter. It is because co-expression of p35 and p40 genes in target cells is required to generate a biologically active p70 heterodimer (Gubler *et al.*, 1991; Schoenhaut *et al.*, 1992). The pNGVL-1 plasmid is a control plasmid, which contains the backbone sequence of pNGVL3-mIL12 without the mIL-12 insert.

Purified Recombinant mIL-12 Protein (rmIL-12) Administration

The purified recombinant mIL-12 protein was a generous gift from the Genetics Institute, Inc. (Cambridge, MA). The rmIL-12 was administered (once per day for 5 consecutive days) into the C57BL/6J mice by intraperitoneal injection in 150 μl PBS containing 0.1% mouse albumin fraction V (Sigma, St. Louis, MO). Control mice were injected with PBS containing the same amount of mouse albumin fraction V.

Hydrodynamics-based Liver Gene Delivery

Plasmid DNA was purified using the plasmid purification kit (Qiagen,

Valencia, CA). Different amount of DNA was diluted in 0.9 % sterilized NaCl and injected intravenously into the mice in a volume of 1.6 ml in about 8 seconds as described (Liu *et al.*, 1999).

ELISA

Mice were bled from the tail vein at various time points after treatment. Serum samples were collected by coagulating the blood at room temperature (2h) or at 4°C (overnight), followed by high speed centrifugation at 14,000 rpm, 4°C for 20 min. Quantitation of mIL-12 was performed using a mIL-12 p70 ELISA kit (R & D Systems, Minneapolis, MN) for the detection of the heterodimer p70. The concentration of p70 in the serum samples was determined from a standard curve with known quantity of p70. Similarly, the production of IFN-γ *in vivo* was assayed using the mouse IFN-γ ELISA kit (R & D Systems, Minneapolis, MN). IFN-γ production in the supernatant from splenocyte cultures was similarly quantitated by ELISA.

NK Activity (YAC-1 Cell Lysis)

Lymph nodes and spleens of C57BL/6J mice were harvested by aseptic technique. Cells from lymph node and spleen were collected by meshing the tissues through a nylon mesh (70 μm). After centrifugation at 1,500 rpm for 5 min, lymphocytes were resuspended in RPMI containing 1x MEM, 0.01 M HEPES, 10

% heat inactivated FBS and 1x antibiotics/antimycotics. Splenocytes were treated with 5 ml RBC lysis buffer (8.32 g NH_4Cl, 0.84 g $NaHCO_3$ and 0.043 g EDTA in 1 L H_2O) for 5 min at room temperature. RBC lysis was neutralized by the addition of 10 ml RPMI. Splenocytes were then centrifuged for 5 min at 1,500 rpm and resuspended with RPMI. YAC-1 cells were radiolabeled with 150 μCi of ^{51}Cr (NEN DuPont, Boston, MA) for 2 h at 37°C with swirling. Radiolabeled cells were then washed for three times in 15 ml of RPMI. Labeled YAC-1 cells (2 x 10^4 cells/well) were incubated with lymphocytes at different ratios in a 96 well plate for 4-5 h at 37°C. YAC-1 cells lysed by 0.1% SDS gave maximal release, whereas cells alone without lymphocytes gave the minimal release. Triplicates or more were set up for each ratio. After incubation, the plate was spun at 1,500 rpm for 5 min and supernatant was collected for gamma counting.

In vitro IFN-γ Release Assay

Spleens were collected from C57BL/6J mice by aseptic technique. Splenocytes were harvested in RPMI as described above and were seeded at a concentration of $5x10^6$/ml in a 24 well plate. After the addition of mIL-12 at different concentrations, the splenocytes were cultured for 48 h at 37°C in 5% CO_2. Supernatant was collected for IFN-γ quantitation with ELISA.

Tumor Model

TC-1 cells (cultured in RPMI), a mouse lung epithelial cell line transformed with HPV-16 genome (Lin *et al.*, 1996), were inoculated subcutaneousely at $1x10^5$ cells/mouse in 6 weeks old C57BL/6J mice (Jackson's Laboratory, Bar Harbor, Maine). TC-1 cells were trypsinized and washed in Hank's buffer for 3 times. At the final washing, cell number and viability of the cells were determined by Trypan blue exclusion. Cells with viability of greater than 80% were inoculated subcutaneously in the flank of the mouse. Tumors were allowed to establish for 9 days before the systemic delivery of naked DNA or rmIL-12 treatment. The size of the tumor was measured every 2-3 days.

Results:

Pharmacokinetics of rmIL-12 and mIL-12 Gene Expression

Pharmacokinetics of mIL-12 gene expression after systemic naked DNA delivery was compared with the pharmacokinetics of rmIL-12 administration after intraperitoneal injection of recombinant protein. Five μg of pNGVL3-mIL-12 plasmid was injected into the mice and serum samples were collected for the detection of circulating mIL-12 levels by ELISA. As reported before (Liu *et al.*, 1999), gene expression mediated by hydrodynamics-based gene transfer was very dependent on the speed of injection. Therefore, the speed of injection was carefully controlled (about 8 sec) for all experiments.

As shown in Fig. 1, at 60 min after injection, a low level of mIL-12 was detected in the serum (about 0.2 ng/ml, n = 5). As short as 5 h after injection, a rapid increase (about 18,000-fold) in the level of mIL-12 production (6.4 ± 1.3 μg/ml, n = 5) was observed. The expression of mIL-12 kept increasing. At 10 h after injection, the level of mIL-12 production was 13.1 ± 3.2 μg/ml (n = 4) and then it reached a maximum level of 22.7 ± 2.7 μg/ml (n = 4) at about 17 h. The systemic administration of the control pNGVL-1 plasmid caused a non-specific production of mIL-12 at a very low (about 1 ng/ml at 5 h) (data not shown). The result showed that liver gene transfer can efficiently produce high levels of mIL-12 *in vivo* and the cytokine is secreted into the circulation. As shown in Fig. 2, the level of mIL-12 production did not decrease until 2 days after injection. There

was an approximate 30-fold reduction in gene expression per day with any given dose of DNA. The apparent half-life of the circulating mIL-12 produced by the gene transfer was estimated to be about 5h.

For the pharmacokinetics study of rmIL-12 distribution, 600 ng of recombinant protein was injected intraperitoneally. Upon injection, a rapid distribution of rmIL-12 in the circulation was observed. As short as 10 min after injection, about 1.3 % of the injected dose was detected in the circulation. At the peak of absorption (about 60 min), 20 % of the injected dose was detected (88 ng/ml equals to a total amount of 114 ng in mice with blood volume of about 1.3 ml). Thereafter, the level of rmIL-12 started to decrease slowly with an apparent half-life (in the elimination phase) of about 205 min. The longer apparent half-life (300 min) of the mIL-12 produced by the systemic gene delivery was probably due to the continuous production of mIL-12 by the liver after the gene transfer.

Effect of Gene Dose on mIL-12 Production

To investigate the effect of gene dose on the production of mIL-12, mice were injected intravenously with different doses of pNGVL3-mIL12 (1 μg, 5 μg and 20 μg). In addition, the time course of expression was also followed at 1, 2 and 4 days after injection (Fig. 2). A general dose-dependent effect of mIL-12 was observed. However, the level of mIL-12 production seemed to reach a maximum with 5 μg of DNA at day 1 as there was no significant difference

between the 5 μg and 20 μg DNA doses. A 13-fold difference in gene expression was observed between the 20 μg and the 1 μg doses. As discussed before, the level of circulating mIL-12 started to drop at day 2 at a rather rapid rate (approximately 30-fold per day). At day 4, the level of mIL-12 was reduced to about 580, 8400 and 17,800 pg/ml for the group treated with 1, 5 and 20 μg of DNA, respectively. With the highest gene dose, a level of about 1,800 pg/ml was still detectable at d7 (data not shown).

NK Cell Activation

IL-12 is known to enhance NK cell activity *in vivo* (Brunda, 1994). In order to study if systemic gene delivery was able to produce functionally active mIL-12, NK cell assay was performed. Cells from lymph node and spleen were collected from mice 6 days after gene transfer. A specific YAC-1 cell lysis was observed with lymph node cells collected from the pNGVL3-mIL12-treated group (Fig. 3). However, no detectable NK activity was observed with splenocytes collected from the same group of mice (data not shown). Lymph node cells from the pNGVL control group only showed minimal background lysis of about 3 %. This mIL-12-mediated NK cell activation demonstrated that gene transfer could produce functionally active mIL-12 *in vivo*.

Stimulation of IFN-γ Production In vivo

Besides the enhancement of NK activity, IL-12 is known to stimulate the production of IFN-γ both *in vivo* and *in vitro*. Moreover, IFN-γ is believed to be one of the major cytokines mediating the antitumor effects of IL-12. Therefore, the time course for the stimulation of IFN-γ production after the gene delivery was followed. Plasmid pNGVL3-mIL12 (5 μg) was administered into the mice. At day 1 after injection, only a minimally detectable level of IFN-γ (98.5 ± 15.7 pg/ml, n = 3) was produced even though the circulating mIL-12 level at this time was maximal (Fig. 4 and Fig. 2). However, injection of pNVGL-1 control plasmid also produced similar level of IFN-γ at day 1. For the pNGVL3-mIL12 group, the level of IFN-γ production rapidly increased by 84-fold (8.3 ± 1.1 ng/ml, n = 3) at day 2 and further increased up to 288-fold (28.4 ± 14.4 ng/ml, n = 3) at day 4. The circulating level of IFN-γ remained high even up to day 7 even though the mIL-12 level was very low at this time (Fig. 1). The control pNGVL-1 only showed a minimal IFN-γ production (Fig. 4).

In vitro IFN-γ Production Induced by mIL-12

In order to compare the activities of mIL-12 produced in mice and the purified rmIL-12, their abilities to induce IFN-γ production in primary splenocyte culture were measured. Circulating mIL-12 was obtained from sera of mice one

day after the injection with pNGVL3-mIL12 and the concentration of mIL-12 in sera was estimated by ELISA. In the concentration range of 10 -10,000 pg/ml, both the gene product in the serum and the rmIL-12 had very similar, if not identical, sigmoidal dose-response curves in inducing the production of IFN-γ at 48 h. In a ^{3}H-thymidine incorporation assay (a common bioassay for IL-12), the mIL-12 gene product was also active in inducing the proliferation of splenocyte culture (data not shown). The results further confirmed that systemic gene delivery could produce large amounts of functionally active mIL-12, which was indistinguishable from rmIL-12.

Antitumor Effect of pNGVL3-mIL12

Since the gene delivery method produced large amounts of mIL-12 with a duration of expression for about 4-7 days, we investigated if systemic mIL-12 gene delivery would show any therapeutic effect in an aggressive cervical carcinoma model. Cervical carcinoma cells (TC-1) were inoculated subcutaneously into C57BL/6J mice. Mice bearing established tumors were treated either with rmIL-12 (0.5 μg/day or 0.2 μg/day for five consecutive days) or a single pNGVL3-mIL12 gene dose (5 μg) delivered by systemic administration. Control plasmid pNGVL-1 was administered in the control group. Growth of tumors in different groups was followed for 32 days (Fig. 6). Twenty-five days after tumor inoculation, mice treated with a single dose of pNGVL3-

mIL12 had 100% tumor regression, which was comparable to the regression (89 %) in the group of mice treated with rmIL-12 (0.5 μg/day for 5 consecutive days). However, for the mice treated with a lower dose of rmIL-12, a partial therapeutic effect was seen (63 % regression at day 25 and later on some tumor reappeared, with a final regression of 25 % at day 32). While mice treated with the control pNGVL-1 plasmid has no tumor regression at any time with the mean (± SEM) tumor size larger than all the mIL-12 treated groups. This result showed that mIL-12 produced by the hydrodynamics-based gene delivery was as potent as the rmIL-12 for the treatment of the TC-1 cervical carcinoma model in mice.

Discussion:

IL-12 is one of the best antitumor cytokines for cancer therapy as it is known to have a variety of immuno-stimulating effects. Its antitumor activity has been demonstrated in a number of tumor models including breast, kidney, ovarian, lung, brain, melanoma and fibrosarcoma (Dias *et al.*, 1998; Sumimoto *et al.*, 1998). However, the antitumor effect of IL-12 is not universal. Resistance to IL-12 therapy has been demonstrated in some acute myeloid leukemia and melanoma models (Nishimura *et al.*, 1996; Ladanyi *et al.*, 1998; Vitale *et al.*, 1998).

The antitumor effect of IL-12 for the treatment of cervical cancer has not been investigated in detail, but some studies implied the potential of IL-12 for cervical cancer therapy. Analysis of biopsy samples from patients with different stages of cervical neoplasia revealed that expression of IL-12, as well as IFN-γ and IL-10, were significantly lower in invasive carcinoma lesions compared with premalignant samples (de Gruijl *et al.*, 1999). Skin transfection of IL-12 potentiates the human papillomavirus E6 DNA vaccine-induced antitumor immune response (Tan *et al.*, 1999). In addition, we have demonstrated earlier that local administration (intratumor injection) of liposome-mIL-12 DNA complexes could cause partial regression of a less aggressive cervical cancer model (C3 cells) (He *et al.*, 1998), indicating the therapeutic potential of IL-12 for cervical cancer or other HPV-positive cancers. In this study, we showed that high level of circulating IL-12 produced by a single gene transfer was sufficient for

complete eradication of established subcutaneous tumors in an aggressive HPV-16 DNA positive cervical carcinoma model (TC-1 cells). The antitumor effect was specific to mIL-12, as the administration of recombinant mIL-12 also resulted in similar antitumor effect. However, repeated injection of a high dose (0.5 μg/day for 5 consecutive days) of rmIL-12 was needed to achieve similar antitumor effect as a single gene transfer. A lower dose of recombinant mIL-12 (0.2 μg/day for 5 consecutive days) only resulted in a partial antitumor response. This indicates that the dose of IL-12 is a determining factor for its antitumor potency.

The production of mIL-12 was accompanied by the induction of high and sustained level of IFN-γ *in vivo* (Fig. 4). Although the mechanisms underlying the antitumor effects of IL-12 have not been fully elucidated, IFN-γ is believed to have an important role in mediating the effect (Brunda *et al.*, 1995). IFN-γ activates innate immunity such as the activation of NK cell and macrophage, which could help eradicating tumor cells. Indeed, mIL-12 gene transfer resulted in the activation of NK cells collected in lymph nodes in our studies (Fig. 3). The reason for undetectable activation of NK cells in lymphocytes collected from spleen was unknown. Besides, IFN-γ can act directly on T and B lymphocytes to promote differentiation. IFN-γ can also enhance both cellular and humoral immunities by inducing the expression of class I and II MHC molecules

(Schendel *et al.*, 2000). In addition, the antitumor effect of IL-12 may also be contributed by the anti-angiogenic activity of IL-12 (Sunamura *et al.*, 2000). Additional mediators other than IFN-γ may be involved in the antitumor activity of IL-12 as the antitumor activity of IL-12 seems to be better and more consistent than IFN-γ (Paillard, 1998).

Our results showed that large amounts of functionally active mIL-12 could be produced by systemic naked DNA delivery. The gene product not only had an antitumor activity comparable with the rmIL-12 (Fig. 6), but also had similar bioactivity as rmIL-12 (Fig. 5). All these results suggested that the gene transfer method used in this study could be useful for rapid screening of IL-12-sensitive tumor, in addition to the cervical carcinoma tested here. It would provide important information for the prediction of responsiveness of different types of cancer to IL-12 therapy.

Recently, we have also reported *in vivo* production of Flt3 ligand by the same delivery approach (He *et al.*, 2000), resulting in a remarkable expansion of dendritic cells in the treated mice. The study demonstrated the usefulness of this systemic gene delivery approach for *in vivo* production of large amounts of gene product. Here, we extended the use of this simple gene delivery method for tumor treatment as a model to demonstrate its powerful potential for *in vivo* assessment of therapeutic effect of a candidate gene. With the advantages of being simple, minimally toxic, highly efficient and economical, the hydrodynamics-based gene

delivery method could become a powerful tool for the study of unknown gene functions *in vivo*. Using this gene delivery method, a comprehensive *in vivo* study of potential therapeutic genes before launching a full-scale preclinical or clinical study would be very helpful.

Many recombinant proteins are being evaluated for the therapeutic effect in animal models, which could be very costly. Although the gene delivery method used in our study cannot be translated for clinical use at the present time (due to its relative invasiveness), it nevertheless demonstrated the utility of the method for assessing potential therapeutic effect of a candidate recombinant protein. This is because the gene product had comparable *in vivo* activity as the recombinant protein. Moreover, this efficient gene transfer method avoids purification, reconstitution, stability and contamination problems for the recombinant proteins. Information obtained from this type of gene transfer study could be very beneficial for clinical trials for recombinant proteins. Besides, a therapeutic recombinant protein could be modified in its gene construct and the *in vivo* screening of its therapeutic effects could be easily assessed by simply delivering the modified gene using this method.

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Legends to Figures:

Fig. 1. Pharmacokinetics of recombinant mIL-12 protein (rmIL-12) and mIL-12 expression in C57BL/6J mice. Six hundred ng of rmIL-12 was injected intraperitoneally. Serum mIL-12 levels were detected at 10, 30, 60, 90, 150, 270 and 420 min after injection (n = 3). Five μg of pNGVL3-mIL12 was administered to the mice by hydrodynamics-based liver gene delivery. Circulating mIL-12 level (p70 heterodimer) was measured by ELISA. Data are shown as mean ± SEM (n ≥ 3).

Fig. 2. A time course study of the gene dose effect on the expression of mIL-12 by systemic delivery of naked DNA. Different amounts of pNGVL3-mIL12 (1, 5 and 20 μg) were injected into each group of mice (n ≥ 4). The circulating level of mIL-12 was measured at day 1, 2 and 4 after gene delivery.

Fig. 3. Activation of natural killer cells (NK) by pNGVL3-mIL12 gene transfer. Specific YAC-1 cell lysis from lymphocytes (collected from lymph nodes) of the treated mice was measured in a ^{51}Cr release assay at day 6 after gene delivery.

Fig. 4. Stimulation of IFN-γ production *in vivo* after pNGVL3-mIL12 gene delivery. Mice (n = 3) were injected with 5 μg of DNA and the level of IFN-γ in the serum at different days after injection was measured by ELISA. IFN-γ

production stimulated by the control pNGVL-1 DNA was also shown.

Fig. 5. Comparison of bioactivities of rmIL-12 and serum mIL-12 produced by pNGVL3-mIL12 injection. Splenocytes from untreated C57BL/6J mice were cultured for 2 days with different concentrations of rmIL-12 (■) or serum mIL-12 (◊) produced after *in vivo* gene transfer (n = 3). Supernatant of the splenocyte cultures were collected for the quantitation of IFN-γ production.

Fig. 6. Comparison of antitumor effects of rmIL-12 administration and pNGVL3-mIL12 gene delivery. Subcutaneous TC-1 tumors were established in C57BL/6J mice. At day 9 after tumor inoculation, the mice in each group ($n \geq 8$) were treated with either rmIL-12 (0.5 μg or 0.2 μg, i.p., for 5 consecutive days) or with a single dose of 5 μg of pNGVL3-mIL12 or control pNGVL-1 plasmid by systemic naked DNA delivery. The size of the non-regressed tumors is shown. The number shown in the bracket is the number of tumors regressed/ total number of tumors inoculated.

Fig. 1

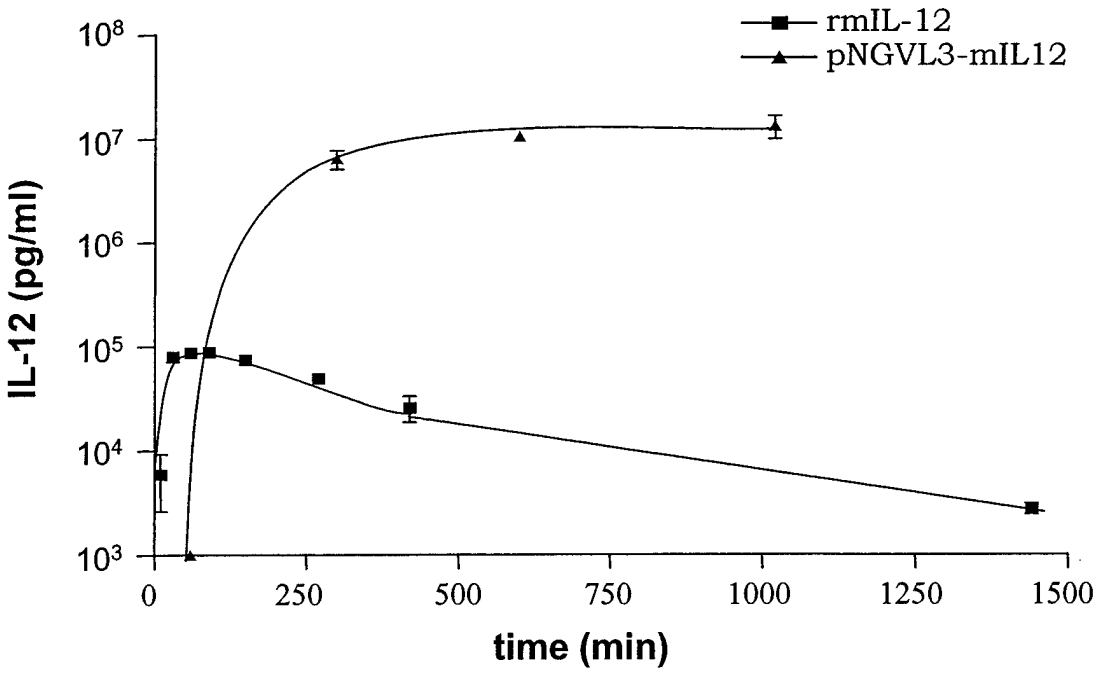

rmIL-12
pNGVL3-mIL12
IL-12 (pg/ml)
10^8
10^7
10^6
10^5
10^4
10^3
0
250
500
750
1000
1250
1500
time (min)

Fig. 2

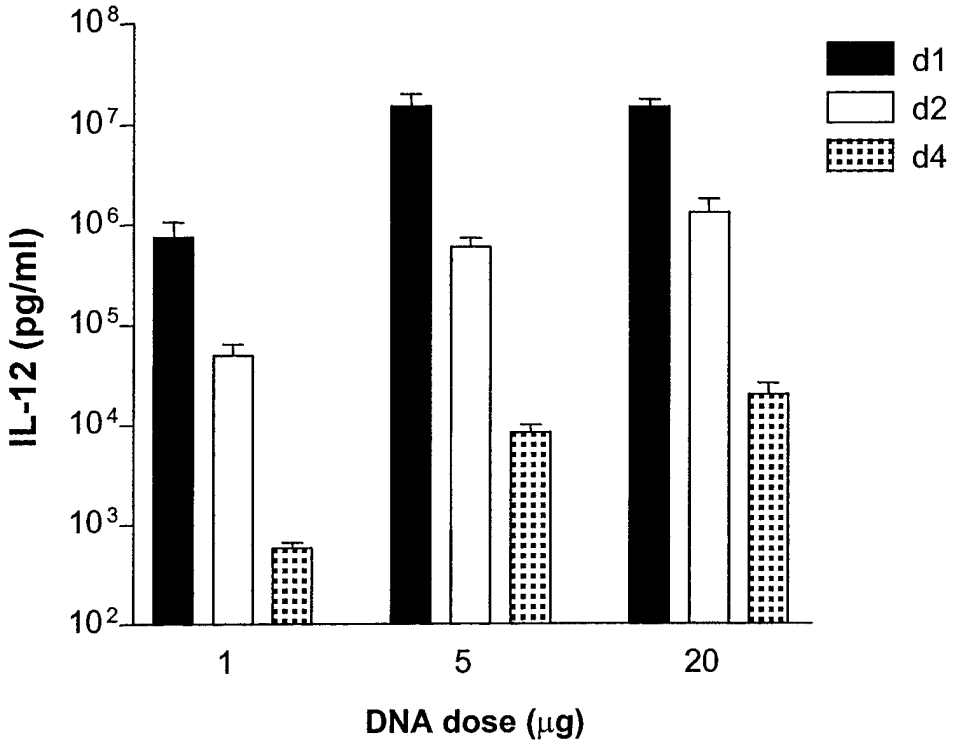


Fig. 3

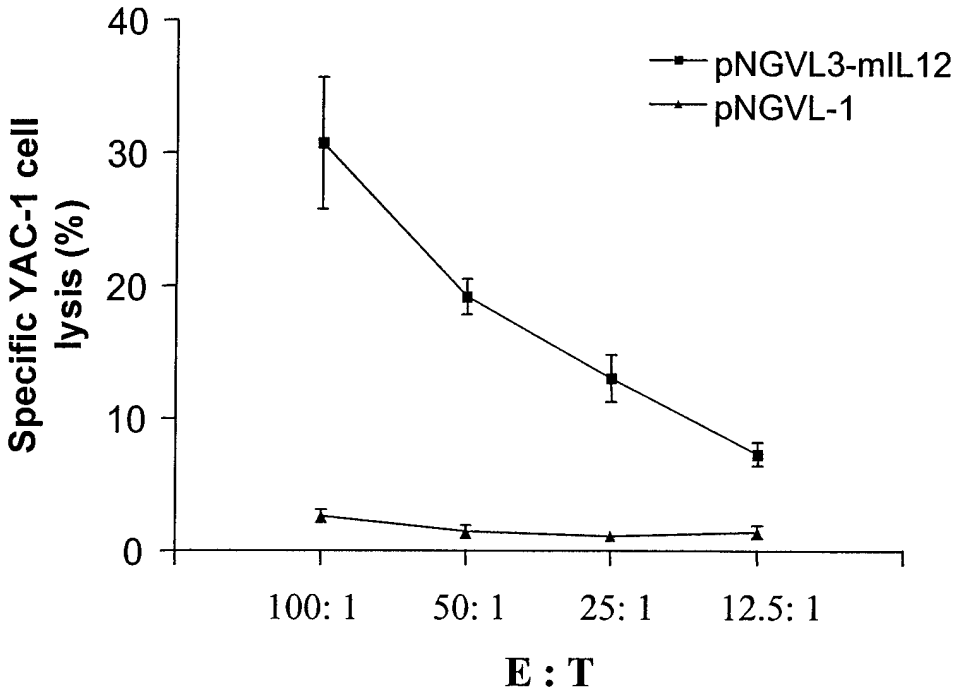

40
30
20
10
0
Specific YAC-1 cell lysis (%)
pNGVL3-mIL12
pNGVL-1
100: 1
50: 1
25: 1
12.5: 1
E : T

Fig. 4

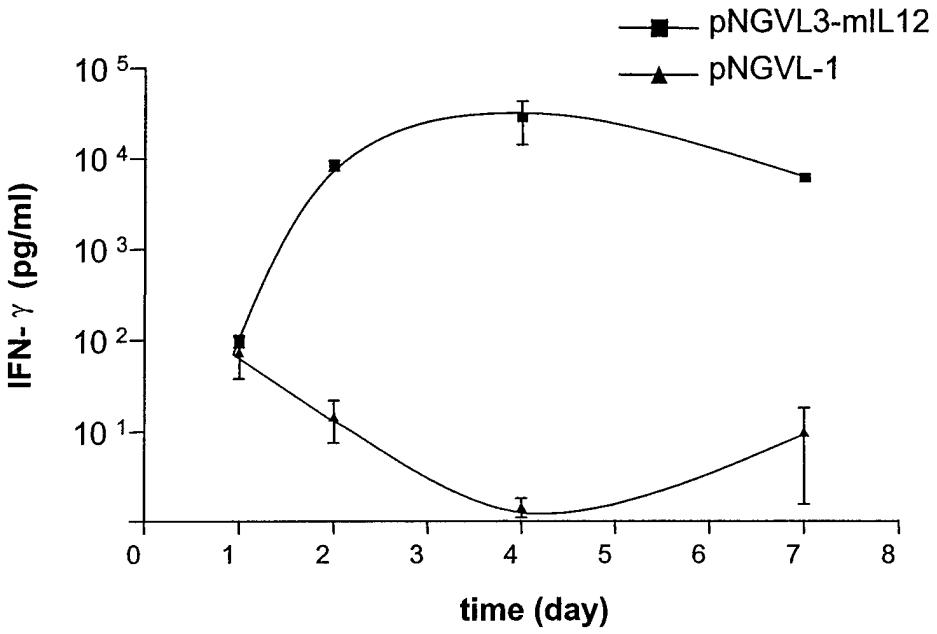

pNGVL3-mIL12
pNGVL-1
IFN-γ (pg/ml)
10 5
10 4
10 3
10 2
10 1
0
1
2
3
4
5
6
7
8
time (day)

Fig. 5

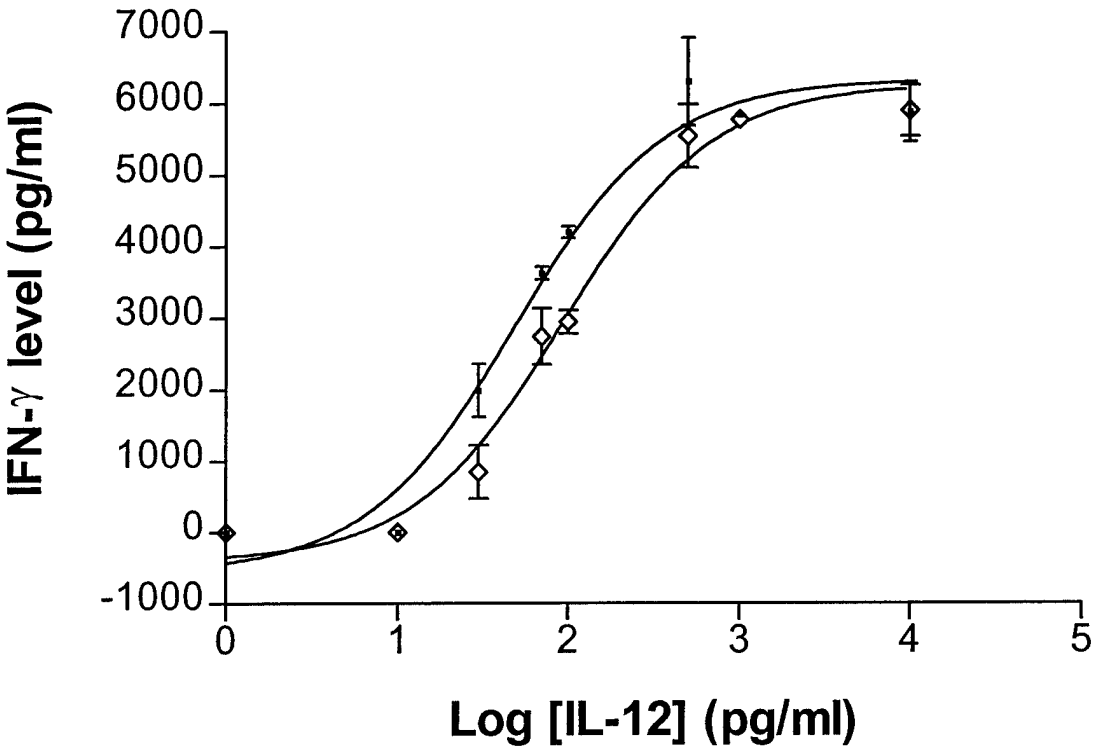
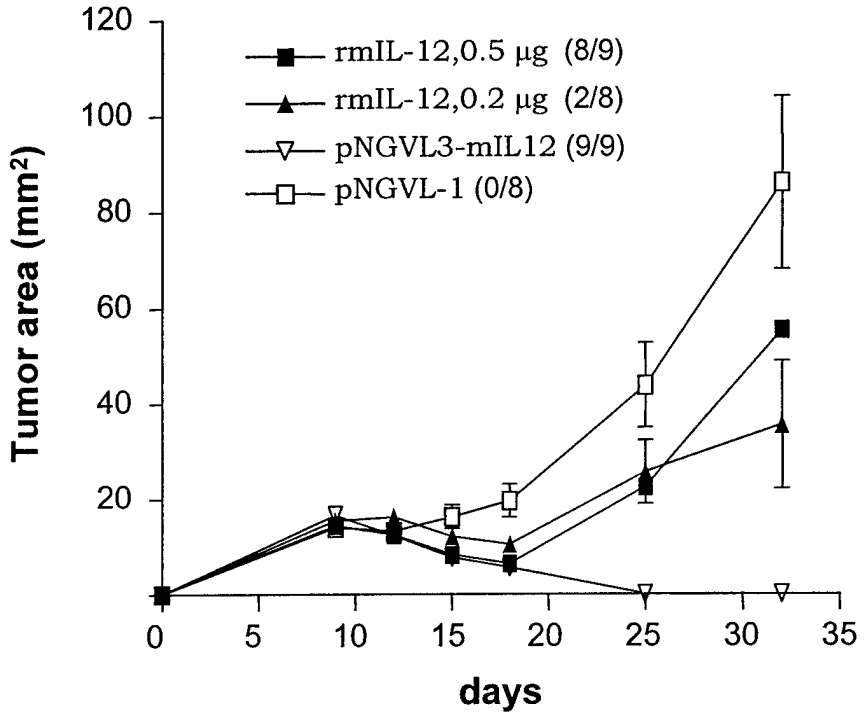

7000
6000
5000
4000
3000
2000
1000
0
-1000
IFN-γ level (pg/ml)
0
1
2
3
4
5
Log [IL-12] (pg/ml)

Fig. 6


120
100
80
60
40
20
Tumor area (mm²)
rmIL-12,0.5 µg (8/9)
rmIL-12,0.2 µg (2/8)
pNGVL3-mIL12 (9/9)
pNGVL-1 (0/8)
0
5
10
15
20
25
30
35
days

Systemic Production of IL-12 by Naked DNA Mediated Gene Transfer: Toxicity and Attenuation of Transgene Expression *In Vivo*

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Abstract:

Background IL-12 is a potent antitumor cytokine for cancer gene therapy. Previously, we demonstrated that single systemic administration of naked DNA could result in profound antitumor effect. In this study, we evaluated the toxicities associated with the IL-12 gene therapy and the effect of initial IL-12 administration on subsequent transgene expression.

Methods Naked DNA was delivered into mice by hydrodynamics-based gene transfer. Liver and systemic toxicity profiles of the mice treated with DNA or recombinant murine IL-12 (rmIL-12) were compared.

Results Systemic administration of naked DNA carrying mIL-12 gene resulted in very similar toxicity as rmIL-12 with respect to liver enzyme, hematological and immunological profiles. Repeated injection of mIL-12 gene did not recover high level of mIL-12 production as the first injection. Moreover, initial mIL-12 administration resulted in inhibition of subsequent reporter gene expression with viral and non-viral promoters (CMV, human α-antitrypsin or chicken β-actin promoter). This transgene inhibition effect was found to be entirely mediated by IFN-γ as the transgene expression was fully recovered in the IFN-γ knockout mice.

Conclusions Systemic IL-12 therapy, with either protein or gene therapy approach, resulted in comparable liver and systemic toxicities. Refractoriness of mIL-12 production by subsequent administation of mIL-12 gene was observed. The transgene attenuation effect of IL-12 predosing, mediated by IFN-γ, provided important insights for the design of IL-12 combination gene therapy and the improvement of gene vectors for IL-12 therapy.

INTRODUCTION

Interleukin-12 (IL-12) is an important therapeutic cytokine demonstrated in a number of murine tumor models (Summoto et al, 1998; Mu et al, 1995). Its multiple stimulatory effects on both innate and cognate cellular immunity make it a potent antitumor agent for cancer immunotherapy (Wolf et al, 1994).

With the promising results obtained from preclinical investigations, a number of clinical trials were undertaken using the purified recombinant human IL-12 protein (rhIL-12) as the therapeutic agent. In the trials, the safety profile, dosing scheme, route of administration and therapeutic effect were found to be encouraging (Robertson et al, 1999; Bajetta et al, 1998, Motzer et al, 1998, Atkins et al, 1997). Complete or partial responses have been reported with some treated patients. The studies also revealed the toxicities associated with the current treatment regimen and the route of administration (local or systemic). Two fatal incidences were reported at the very first clinical trial with systemic administration (Marshall, 1995). The mechanisms underlying the IL-12 toxicities are not fully understood. However, interferon-γ (IFN-γ) is believed to be one of the mediators. In summary, the toxicities include transient increase in transaminases, high level of IFN-γ, lymphopenia, hypoalbuminemia, anemia, etc. These initial clinical results demand the improvement of IL-12 delivery method, optimization of dosing regimen and the prevention of IL-12 associated toxicities. Recently, several studies demonstrated the feasibility of reducing the IL-12 associated toxicities. For instance, a single IL-12 predosing was found to exert some protective effects against the IL-12 toxicity (Leonard et al, 1997) and erythropoietin was found to be effective for the prevention of IL-12 induced anemia and thrombocytopenia without interfering with the antiumor activity in

mice (Golab et al, 1998).

With the promising preclinical outcome, IL-12 has become an attractive candidate for the cancer gene therapy. Recently, various viral and non-viral vectors have been developed successfully for animal therapy studies (Tan et al, 1996; Parker et al, 2000; Puisieux, 1998; Paul et al, 2000). However, the dosing regimen and the toxicity associated with IL-12 gene therapy have not been carefully investigated. Since the toxicity issue of IL-12 therapy is of important concern, the evaluation of toxicity profiles for IL-12 gene vectors should not be overlooked. It is especially important for viral vectors because high antigenicity and inflammatory activity of the vector may further complicate the toxicity issue of IL-12 therapy. Non-viral vectors are better choices for the delivery of a cytokine gene as they are less immunogenic than viral vectors. However, the efficiency of non-viral vector is still the main limitation. Repeated administration and prolonging gene expression by vector modifications are common alternatives to improve the efficiency non-viral vectors. Thus, optimization of dosing regimen of non-viral IL-12 vector administration becomes necessary.

IL-12 is very potent when delivered as a single therapeutic agent. Nevertheless, the response rate for different kinds of tumor is not always 100 %. Therefore, the effects of combination therapy of IL-12 with additional therapeutic genes or chemotherapy have been investigated. Combination IL-12 therapies are aimed at improving the therapeutic efficacy and reducing the IL-12 associated toxicity (Nakamura, 2000). Some combination therapies with IL-12 have demonstrated improved therapeutic effects in animals (He et al, 1998). As cytokines are known to have complicated regulatory activity, combination therapy with cytokines has to be carefully studied. For instance, some cytokines (IFN-γ,

IFN-α and tumor necrosis factor-α) show inhibitory effects on the expression of transgene carrying viral promoters (Qin et al, 1997; Harms et al, 1995). Therefore, it is particularly important to study any possible effects of IL-12 on additional transgene expression in the context of combination gene therapy.

Previously, we demonstrated that a single systemic administration of naked DNA encoding murine IL-12 gene (mIL-12) could result in profound antitumor effect in a cervical carcinoma model. In order to see if this gene transfer method is a good model for preclinical studies of IL-12 toxicity, we evaluated the toxicities associated with this mIL-12 gene transfer method in comparison with the recombinant murine IL-12 protein (rmIL-12). The effect of repeated injection on mIL-12 production was studied. In addition, we investigated the effect of initial mIL-12 administration on subsequent transgene expression.

MATERIALS AND METHODS

Plasmid Constructs

pNGVL3-mIL12 (also named as pCMV-mIL12 in this study for the indication of CMV promoter) and the pNGVL-1 plasmids were kind gifts from the National Gene Vector Laboratory at the University of Michigan. The pNGVL3-mIL12 plasmid carries the murine p35, an internal ribosome entry site and the p40 gene driven by a single CMV promoter. Co-expression of p35 and p40 genes in target cells is required to generate a biologically active p70 heterodimer (Gubler et al., 1991; Schoenhaut et al., 1992). The

control pNGVL-1 plasmid contained the backbone sequence of pCMV-mIL12, but without the mIL-12 insert.

pAAT-Luc plasmid (with a human α1-antitrypsin promoter driving the expression of luciferase gene) was cloned as followed: the AAT promoter fragment was obtained by digesting pAAT-P(+) (a kind gift from Dr. Xiao Xiao, University of Pittsburgh, PA) with *Kpn* I and *Xba* I. The pGL3-Basic vector (Promega, Madison, WI) was digested with *Kpn* I and *Nhe* I. Then, the AAT promoter fragment was ligated to the pGL3-Basic vector to generate pAAT-Luc. The activity of pAAT-Luc was confirmed by luciferase gene expression in transfected cells.

pAAT-mIL12 plasmid was constructed from pAAT-Luc. Briefly, mIL-12 gene was PCR amplified with primers carrying *Sma* I and *Nhe* I sites. The PCR product was digested with *Sma* I and *Nhe* I. The pAAT-Luc was then digested with *Sma* I and *Xba* I to remove the luciferase gene. Then, the backbone fragment carrying the AAT promoter was isolated and used for ligation with the mIL-12 PCR fragment. The pAAT-mIL-12 clone was confirmed by restriction digestion and the function of the gene was tested by injection of the plasmid into mice and assayed for mIL-12 by ELISA. The chicken β-actin-Luc (pCba-Luc) was constructed by cloning a PCR fragment carrying the chicken β-actin promoter between the *Nhe* I and *Sma* I sites in front of the luciferase gene in the pGL3-Basic vector. Plasmid DNA was purified using the plasmid purification kit (Qiagen, Valencia, CA).

Hydrodynamics-based Gene Transfer

Five μg of purified plasmid DNA was diluted in 0.9 % sterilized NaCl and

injected intravenously into the mice in a volume of 1.6 ml in about 8 seconds as described (Liu et al, 1999).

Purified Recombinant mIL-12 protein (rmIL-12) Administration

Purified recombinant mIL-12 protein was a generous gift from the Genetics Institute Inc. (Cmabridge, MA.). The rmIL-12 (0.5 or 1 μg) was administered into mice by intraperitoneal injection in a 150 μl volume of PBS containing 0.1% mouse albumin fraction V (Sigma, St. Louis, MO). Control mice were injected with PBS containing 0.1% mouse albumin fraction V.

Animals

Female C57BL/6J mice and C57BL.6J ifng ^{tm1ts} transgenic mice (interferon-γ knockout mice) at 6-8 weeks were used (Jackson's Laboratory, Bar Harbor, Maine). Animals were kept at the animal facility at the University of Pittsburgh, PA.

Toxicity Study

Mice were treated either with 5 μg pCMV-mIL12 or pNGVL-1 by i.v. injection. Another group of mice was treated with 0.5 μg rmIL-12 or PBS containing 0.1 % mouse albumin fraction V for 5 consecutive days by i.p. injection. Blood and serum samples from the gene-treated mice were collected at day 1, 2, 4, 6, 8, 12, 16, 23 and 30 after the injection. However, for mice treated with rmIL-12 or PBS control, blood and serum samples were only collected at day 1, 2, 4, 6, 8, 12, 16, 23 and 30 after the last i.p. injection. At different days after treatment, mice were anesthetized with isoflurane and

blood samples were collected by retro-orbital bleeding. Blood samples were collected in EDTA. Serum samples were obtained by first collecting blood samples in tubes containing serum separator, followed by aggregation of blood at room temperature for 30 min and then centrifugation at 14,000 rpm for 2 min. Samples were analyzed by Antech Diagnostics (Farmingdale, NY). The sample collection tubes were provided by Antech Diagnostics.

IL-12 ELISA

Mice were bled from the tail vein at various time points. Serum samples were collected by coagulating the blood at room temperature (2 h) or at 4°C (overnight), followed by high-speed centrifugation at 14,000 rpm, 4°C for 20 min. Quantitation of mIL-12 was performed using a mIL-12 ELISA kit (R & D Systems, Minneapolis, MN) for the detection of the heterodimer p70. The concentration of p70 in the serum samples was determined from a standard curve constructed with known concentrations of p70.

Luciferase assay

Mice were sacrificed and liver, lung and heart were collected. Luciferase lysis buffer (1 ml) containing 0.05 % Triton X-100, 2 mM EDTA and 0.1 M Tris-HCl at pH 7.8 was added to the samples on ice. Tissue samples were then homogenized with a tissue tearor on ice. Homogenized samples were then centrifuged at 14,000 rpm for 5 min at 4 °C. Supernatant was collected for luciferase assay using the luciferase assay kit from Promega (Madison, WI). Luminescence was measured with a luminometer (Wallac Inc., Gaithersburgh, MD). Luciferase activity was normalized as relative light units per mg of

tissue protein in the supernatant (RLU/mg protein).

Protein Assay

Protein concentration in the supernatant after lysis was determined using the Coomassie Plus protein reagent (Pierce, Rockford, IL) using bovine serum albumin as standards. Absorption at 595 nm was measured. Protein concentration in the samples was calculated from the standard curve.

RESULTS

Toxicity Comparison between mIL-12 Gene Delivery and rmIL-12

The toxicities associated with the systemic mIL-12 gene transfer and recombinant mIL-12 administration were extensively compared. This included the analyses of liver profile (alkaline phosphatase, ALT, AST, bilirubin, albumin and total protein levels), hematological profile (hemoglobin level, hematocrit and red blood cell number), complete blood count (absolute counts of platelets, polymorphonuclear cells, lymphocytes, monocytes, eosinophils and basophils) and creatinine level.

Mice were injected with a single gene dose of 5 μg DNA (pCMV-mIL12 or pNGVL-1) at day 1. Blood and serum samples were collected at 1, 2, 4, 6, 8, 12, 23 and 30 days post-injection. For mice treated with rmIL-12 (0.5 μg/day, i.p. for 5 consecutive days) or control vehicle (albumin), samples were collected only at 1, 2, 4, 6, 8, 12, 23 and 30 days after the last injection (equivalent to day 6, 7, 9, 11, 13, 17, 28 and 35 after the

first injection).

Changes in Liver Profile

The effect of mIL-12 gene transfer on liver function was investigated (Table 1). Liver enzyme levels of the untreated mice collected at day 1 were used as reference. The administration of pCMV-mIL12 resulted in transient and progressive decrease in alkaline phosphatase level. The level was reduced by 60 % of the untreated control at day 8 and then slowly recovered. Similarly, the alkaline phosphatase level of the rmIL-12-treated group was reduced by 60 % of the control at day 2 (equivalent to day 7 after the first rmIL-12 injection). For the respective control group of each treatment (i.e. pNGVL-1 and albumin groups), the level of alkaline phosphatase remained basically unchanged, except at the very late points. The reason for this non-specific decrease was unknown.

Serum levels of AST and ALT are indicators of liver damage. We observed that the systemic administration of naked DNA (either pCMV-mIL12 or pNGVL-1) caused transient liver damage as indicated in the abrupt and marked increase of both AST and ALT levels in the first 2 days. The AST and ALT levels in the control pNGVL-1 group recovered at about day 6, while the levels of the pCMV-mIL12 group were still elevated at later time points. This damage might due to the sustained production of low level of mIL-12 by the gene transfer. The rmIL-12 caused a similar increase in AST and ALT levels but with a smaller magnitude and shorter duration. In addition, levels of total protein and albumin in the blood also dropped transiently and recovered by both forms of mIL-12 treatments (data not shown). The results demonstrated that systemic mIL-12 administration, either in the form of a gene or recombinant protein, could cause transient

but reversible liver damage as indicated by the changes in liver enzyme levels.

Hematological Effects

Examination of blood chemistry revealed that both the systemic mIL-12 gene delivery and the rmIL-12 caused a severe reduction in hematocrit of about 50 % of the untreated control, while the respective controls showed no changes (Table 2). Similar reduction in red blood cell number was also observed (data not shown). This mIL-12-specific effect was reversible. Similar mIL-12 specific changes were observed for hemoglobin content of blood (reduction of 60 % compared to the untreated reference) in both mIL-12 treated groups. In the control groups, a reduction in hemoglobin content at late time points was observed. The reason for the reduction was unknown.

Effects on Leukocyte Subsets

As shown in Fig. 1, both the administration of mIL-12 gene and the rmIL-12 resulted in an initial reduction (from day 1 to about day 4) in lymphocyte number accompanied by an increase in polymorphonuclear cells (PMN) in the blood. After these initial changes, the lymphocyte and PMN numbers remained rather similar to the respective controls. Other indicators of toxicity, e.g. bilirubin, globulin and creatinine, were not significantly altered (data not shown).

Repeated Injection of pCMV-mIL12

An initial injection of 5 μg of pCMV-mIL12 resulted in the production of high level of mIL-12 (3.97 ± 0.73 μg/ml) in the circulation (Fig. 2). However, this initial

injection of pCMV-mIL12 resulted in a prolonged non-responsive period to second injection. Even 12 days after the first injection, the second injection of mIL-12 DNA was still unable to reach a level of mIL-12 production comparable to the first one.

Effect of Pre-injection on Liver Function

We tested if the liver was damaged by initial injection of DNA, thus resulting in the impaired expression of the subsequently delivered gene. Mice were initially (day 0) injected with 5 μg of either pCMV-Luc, pNGVL-1 control plasmid or saline alone. At day 4, the mice were then injected with pCMV-Luc again and the level of gene expression was monitored 24 h later. As shown in Fig. 3, the group treated with a single pCMV-Luc injection at day 0 still retained an appreciable but low level of luciferase gene expression at day 5. This was due to the decline in gene expression over time. When compared with the saline pre-injected group, pre-injection of plasmid DNA (either pCMV-Luc of pNGVL-1 control) resulted in the same level of gene expression in the liver upon the second injection of pCMV-Luc. Similar results were obtained when the interval of injection was increased to 6 days (data not shown). Therefore, pre-injection of a control plasmid DNA did not result in impaired function of liver for gene expression.

Impaired mIL-12 Production with CMV or AAT Promoter

Then, we investigated if this expression inhibition was specific to CMV promoter. We had this concern because CMV promoter and many other viral promoters are inhibited by IFN-γ (Qin et al, 1997; Harms et al, 1995), which is stimulated by IL-12. Mice were initially injected with pCMV-mIL12. Four days later, either pCMV-mIL12 or

pAAT-mIL12 (containing a liver specific human α1-antitrypsin promoter) was administered (Fig. 4). Serum mIL-12 level was monitored 24 h after each injection. The pAAT-mIL-12 plasmid was functional as the expression of mIL-12 from pAAT-mIL12 was comparable with that from pCMV-mIL12. By using a non-viral AAT promoter in the second injection, the circulating mIL-12 level was still about 3 orders of magnitude lower than the level achievable by the first pCMV-mIL12 injection (Fig. 4). Thus, the inhibition of mIL-12 gene expression from the second injection was not specific for CMV promoter only, but also for AAT promoter.

Transgene Inhibition for Viral and Non-viral Promoters

From the above observations, we hypothesized that initial mIL-12 administration would have an inhibitory effect on the expression of the subsequently delivered gene (either with the same or a different gene), despite the kind of promoters involved. To test the hypothesis, mice were first injected with either pCMV-mIL12 or pNGVL-1 control. Four days later, mice were then re-injected with different luciferase plasmid vectors carrying CMV promoter (pCMV-Luc), AAT promoter (pAAT-Luc) or chicken β-actin promoter (pCba-Luc). Pre-injection of mIL-12 gene greatly inhibited luciferase expression in the liver in all 3 cases (Fig. 5). Compared to the pNGVL-1 pre-injected control, the expression of luciferase in the liver for pCMV-Luc, pAAT-Luc and pCba-Luc was reduced by 41-, 25- and 203-fold, respectively. For the plasmid carrying the strongest promoter of the three, pCMV-Luc, the luciferase expression in the lung and heart were also reduced by about 5-fold compared to the pNGVL-1 control (data not shown). As AAT and Cba are weaker promoters, the expression of luciferase in organs

other than the liver was low and the changes in these organs were not detectable.

Transgene Inhibition by rmIL-12

The attenuation effect of transgene expression by initial injection of mIL-12 gene was further confirmed in an experiment with pretreatment of rmIL-12. Mice were pretreated with either the vector albumin or rmIL-12 (1 μg/day for 2 days). On the third day, different luciferase plamids were administered. As shown in Fig. 6A, pretreatment of mice with recombinant mIL-12 protein also resulted in a marked transgene inhibition with all luciferase plamids. For pCMV-Luc, pAAT-Luc and pCba-Luc, the expression in the liver was reduced by 26-, 61- and 74-fold, respectively, as compared to the albumin control. The expression of pCMV-Luc in lung and heart were also reduced by 21- and 14-fold (data not shown). Thus, mIL-12 had a specific attenuation effect on the expression of a subsequently delivered gene and the attenuation was observed in all three viral and non-viral promoters tested.

Role of IFN-γ

IFN-γ has been shown to inhibit gene expression from many viral promoters. In order to understand the underlying mechanism of the transgene attenuation effect of mIL-12, we tested if IFN-γ was responsible for this effect. IFN-γ knockout mice (C57BL/6J-ifng ^{tm1ts}) with the same genetic background as C57/BL6J mice were used. Experiments were performed as before with the rmIL-12 pretreatment (Fig. 6B). Interestingly, the transgene attenuation effect induced by mIL-12 pretreatment was completely lost in the IFN-γ knockout mice and the level of reporter gene expression from different promoters

(CMV, AAT or Cba) was the same as the control group. This observation strongly suggested that IFN-γ signaling was mainly responsible for the transgene attenuation effect of mIL-12 predosing.

DISCUSSION

We have previously shown that efficient mIL-12 gene transfer can be achieved by systemic administration of naked DNA. A single administration of mIL-12 gene resulted in the production of high level of mIL-12, which was sufficient to exert a profound therapeutic effect on established tumors in a cervical carcinoma animal model. Here, we evaluated the toxicities associated with this gene transfer method in comparison with recombinant mIL-12 protein administration.

The main organ for transfection by the hydrodynamics-based gene transfer is the liver (Liu et al, 1999), which would be mainly responsible for mIL-12 production after gene transfer. Therefore, we investigated if there was any liver damage associated with the high local concentration of mIL-12 produced upon gene transfer. In general, transient changes were observed for liver enzymes (alkaline phosphatase, AST and ALT) and albumin levels in both the gene-treated and the rmIL-12 treated groups indicating liver injury. However, the systemic gene delivery method seemed to introduce a more abrupt liver damage than the rmIL-12 in the first two days after gene transfer. The changes in liver profile introduced by mIL-12 gene transfer lasted longer compared with that induced by rmIL-12. This may be contributed by the prolonged expression of mIL-12 in the liver. In fact, a sustained low level of mIL-12 was still detectable 7 days after

injection (unpublished data). Hematological profiles and the changes in leukocyte subsets for the gene-treated and rmIL-12-treated mice were very similar. Dramatic but reversible reduction of hematocrit, hemoglobin content and red blood cell numbers were observed. In addition, a moderate and transient leukopenia accompanied by an increase in PMN was observed for both treatment groups. We concluded that the systemic administration of DNA and the rmIL-12 have similar and comparable toxicity profiles. Since the underlying mechanisms for IL-12 toxicity are not fully understood, therefore, this gene delivery method could be used to establish a good model for the mechanistic study of IL-12 induced toxicities.

We also investigated the effect of repeated systemic administration of mIL-12 gene. Surprisingly, the initial administration of pCMV-mIL12 was found to result in a prolonged non-responsive period to the second injection. Even up to 12 days, the second gene transfer seemed non-effective in producing mIL-12 at a level comparable to the first one. This was different from our previous finding that repeated injection of another cytokine (Flt3-ligand) could achieve the same level of cytokine production as the first injection (He et al, 2000). The followings are some possible explanations for the observation: a) the liver was damaged by mIL-12 associated mechanisms and became refractory for uptake and expression of any DNA; b) CMV promoter was inhibited by mIL-12; c) attenuation of mIL-12 expression was at transcriptional or translational level; d) the secretion of mIL-12 into the circulation was inhibited; e) the mIL-12 predosing somehow triggered rapid degradation or clearance of mIL-12 produced by subsequent injection; f) mIL-12 may have an auto-feedback mechanism that inhibit the production of excessive amount of mIL-12. Some of the possibilities were investigated.

The first possibility was experimentally ruled out as repeated injection of a reporter luciferase gene was able to achieve the same level of expression as the first injection (Fig. 3). Some cytokines, like TNF-α, IFN-α and IFN-γ (IFN-γ is stimulated by IL-12), have been shown to have inhibition on many viral promoters including CMV promoter. Therefore, the possibility of CMV promoter attenuation was investigated. However, the observed refractoriness of mIL-12 production with pAAT-mIL12 (a human liver specific promoter) at the second injection suggested that this effect was not specific to the CMV promoter.

From these observations, we hypothesized that initial mIL-12 administration would have an inhibitory effect on the expression of the subsequently delivered gene (either the same or a different gene), despite the kind of promoters involved. We tested the hypothesis by predosing the mice with either mIL-12 gene or rmIL-12 and then the expression of reporter luciferase plasmids carrying different promoters delivered subsequently was followed. Interestingly, for all three viral promoter and non-viral promoters tested (CMV, AAT and Cba), luciferase expression was greatly inhibited (even up to 200 fold) (Fig. 5 and Fig. 6A). More importantly, the full recovery of transgene expression (with all three promoters) in the IFN-γ knockout mice strongly supported that IFN-γ signaling was mainly responsible for the transgene attenuation effect of mIL-12 predosing (Fig. 6B). In fact, this was supported by our earlier finding that high level of IFN-γ was produced after the mIL-12 gene transfer (unpublished data).

IFN-γ is an antiviral and anti-inflammatory cytokine. Recently, it has been shown to inhibit gene expression from many viral promoters *in vitro* (Qin et al, 1997; Harms et al, 1995). It is believed to be one of the major mechanisms involved in the host protection

against viral infection. Recently, Qin et al (1997) showed that the inhibition of expression from the viral promoters occurred at the mRNA level. However, we found that IFN-γ seemed to have a strong inhibition effect on both viral and non-viral promoters (Fig. 5 and Fig. 6). In agreement with our observation was that retrovirus-mediated transgene expression from a cellular promoter (involucrin) was also inhibited by IFN-γ (Ghazizadeh et al, 1997). This rather general effect of IFN-γ on exogenous gene expression is likely to be mediated through some common mechanisms with broad range of effects. One possibility will be the interference of RNA stability. IFN-γ is known to induce the activation of RNase L, which is involved in the degradation of double stranded RNA (Zhou et al, 1997; Cebulla, 1999). The possible involvement of RNase L could be tested by down-regulating its gene expression using antisense. The second possibility is that IFN-γ production may induce or reduce the expression of cellular proteins, which could either result in reduced expression of transgene or degradation of transgene product. The third possibility is the induction of necrosis or apoptosis in different organs by the systemic effect of IFN-γ (Kano et al, 1999), which may render the inability to express exogenous gene in these organs.

The findings of this study have the following significant implications. First, we previously observed that IFN-γ was partially responsible for the refractoriness of LPD (liposome-/protamine/DNA complexes) injection (Li et al, 1999). Moreover, pretreatment with an anti-inflammatory drug, dexamethasone, was able to prolong in vivo gene expression from non-viral vectors (Tan et al, 1999). Our observation in this study confirmed the importance of IFN-γ in mediating the shutdown of non-viral gene expression. Interestingly, the effect was also observed in retroviral gene transfer

(Ghazizadeh et al, 1997). Our results may provide insights for the design of non-viral vector (and viral vector) with prolonged expression. For instance, IFN-γ inducible promoter or promoter modified with IFN-γ responsive element may be used to prolong gene expression. Secondly, gene therapy with IL-12 as a single agent or in combination needs to be carefully monitored for its effectiveness and the dosing schedule has to be optimized. This is because the transgene attenuation effect of IL-12 may affect the efficacy of IL-12 gene therapy or any combination therapies involving the use of recombinant IL-12 or IL-12 gene with addition therapeutic genes.

A better understanding of the mechanisms underlying the systemic IL-12 toxicities including the refractoriness of repeated systemic mIL-12 gene injection, and attenuation of transgene expression by mIL-12 predosing would definitely provide useful insights for the design of IL-12 combination gene therapy, for the improvement of both viral gene and non-viral vectors for gene therapy, including IL-12 gene therapy.

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Legends to the Tables:

Table 1. Liver toxicity was followed at different days after mIL-12 treatments. Changes in enzyme levels were monitored for the mIL-12 gene transfer and the rmIL-12 administration ($n \geq 3$). The respective controls, NGVL-1 and albumin, were included ($n \geq 3$). For mIL-12 gene transfer, d1 refers to the first day after the gene transfer, while for rmIL-12 administration, d1 refers to the first day after the last injection of the 5-consecutive day dosing regimen (i.e. equivalent to day 6 after the first injection). The mean % change compared with untreated mice at d1 ($n \geq 7$) was calculated as: (*x-untreated*) x 100 % / *untreated*. AST: aspartate aminotransferase, ALT: alanine aminotrasnferase.

Table 2. Comparison of hematological changes between the mIL-12 gene transfer and the rmIL-12 administration ($n \geq 3$). The mean % change compared with untreated mice at d1 was calculated as: (*x-untreated*) x 100 % / *untreated* ($n \geq 7$). For mIL-12 gene transfer, d1 refers to the first day after the gene transfer, while for rmIL-12 administration, d1 refers to the first day after the last injection of the 5-consecutive day dosing regimen (i.e. equivalent to day 6 after the first injection).

Legends to the Figures:

Fig. 1. Changes in leukocyte subsets by mIL-12 treatments. The percentage change in polymorphonuclear cells (PMN) and lymphocytes (L) were indicated. Panel A shows the changes after pCMV-mIL12 transfer. Panel B shows the changes occurred after rmIL-12 treatment ($n \geq 3$).

Fig. 2. Initial mIL-12 gene transfer resulted in refractoriness to mIL-12 production by second injection. Serum IL-12 (p70) level was monitored at 24 hrs after each injection. The first injection was given at d0. At d4, d6 and d12 after the first injection, second injection of 5 μg of pCMV-mIL12 was given ($n \geq 3$).

Fig. 3. Effect of DNA pre-injection on liver function. Mice were first injected with 5 μg of pCMV-Luc, pNGVL-1 or NaCl (0.9 %) at d0. Four days later, either no injection or a second injection of pCMV-Luc was given ($n \geq 3$).

Fig. 4. Impaired production of mIL-12 with CMV and AAT promoters by initial mIL-12 gene transfer. Mice were injected with 5 μg of pCMV-mIL12 at d0. Four days later, a second injection with pCMV-mIL12 or pAAT-mIL12 was given ($n \geq 4$). The serum IL-12 level was monitored at 24 hrs after each injection.

Fig. 5. Attenuation of transgene expression by initial mIL-12 gene transfer. At the first injection, mice were either injected with 5 μg of pNGVL-1 control or pCMV-mIL12. Four days later, luciferase plasmids with different promoters (CMV, AAT or Cba

promoter) were injected by systemic naked DNA injection ($n \geq 4$). The luciferase expression in the liver is shown.

Fig. 6. Attenuation of transgene expression by rmIL-12 pretreatment (Panel A). Mice were pretreated with rmIL-12 (1 μg/day for 2 days, i.p.). On day 3, luciferase plasmids with different promoters (CMV, AAT or Cba promoter) were injected ($n \geq 4$). The luciferase expression in the liver is shown. IFN-γ signaling was responsible for the transgene attenuation effect of initial rmIL-12 injection (Panel B). IFN-γ knockout mice (C57BL.6J ifng ^{tm1ts}) were pretreated with rmIL-12 (1 μg/day for 2 days, i.p.). On day 3, luciferase plasmids with different promoters (CMV, AAT or Cba promoter) were injected ($n \geq 4$). The luciferase expression in the liver is shown.

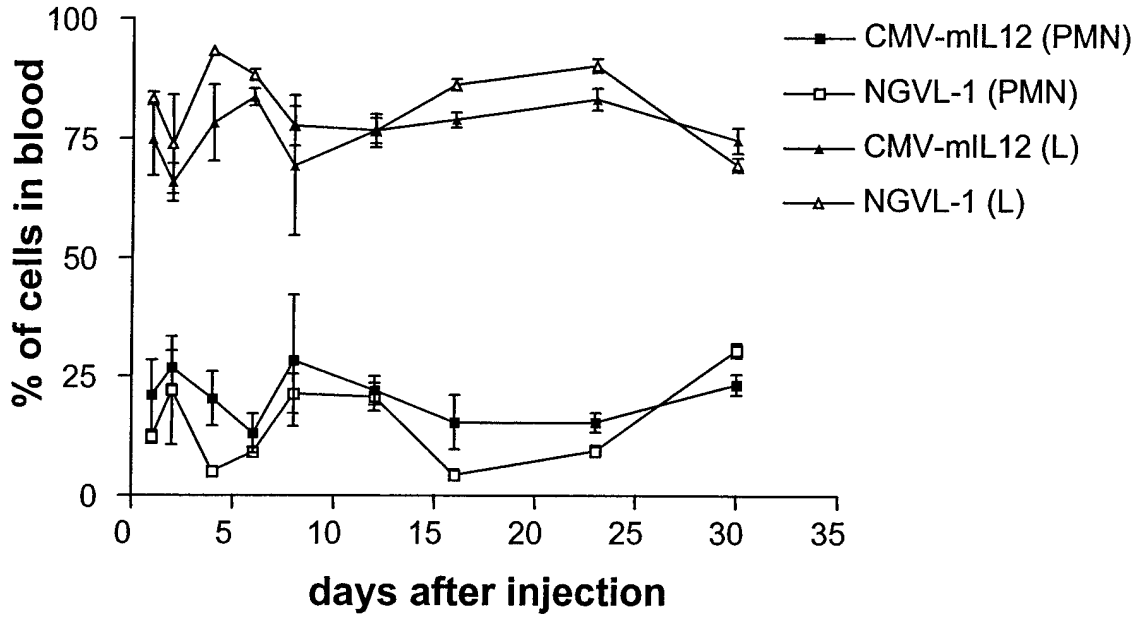
Table 1. Comparison of liver enzyme profiles after mIL-12 gene transfer and rmIL-12 administration.

Liver enzyme (IU/L)	Treatment	Mean % change compared with untreated at d1								
		d1	d2	d4	d6	d8	d12	d16	d23	d30
Alkaline Phosphatase	pNGVL3-mIL12	17.8	0.5	-14.5	-47.0	-60.9	-33.5	-14.8	27.2	1.6
	pNGVL-1	-9.2	-4.4	1.5	11.0	2.6	16.0	-8.4	-16.3	-37.8
	rmIL-12	-57.0	-60.5	-54.5	-42.1	-31.3	26.5	2.9	2.9	-19.8
	Albumin	-1.6	11.8	7.8	6.6	2.5	-2.1	-21.4	-21.3	-40.2
AST	pNGVL3-mIL12	429.9	221.5	125.5	82.8	116.5	116.2	28.9	60.3	57.7
	pNGVL-1	566.8	365.2	50.6	-10.7	2.4	21.2	34.4	3.8	102.8
	rmIL-12	125.1	247.4	237.8	-1.4	82.6	-10.4	36.5	-18.9	-6.7
	Albumin	21.3	8.9	8.9	17.7	28.1	9.1	-6.5	11.0	-3.5
ALT	pNGVL3-mIL12	2562.7	633.5	336.2	345.5	310.3	621.2	276.2	143.4	137.8
	pNGVL-1	3233.4	1355.8	19.7	-9.6	-6.2	0.5	-4.0	-10.7	12.2
	rmIL-12	212.3	624.3	245.0	90.1	97.6	-8.5	-2.0	-2.0	-11.2
	Albumin	-10.4	-14.6	-18.8	32.3	-8.7	-28.0	-12.1	1.3	3.8

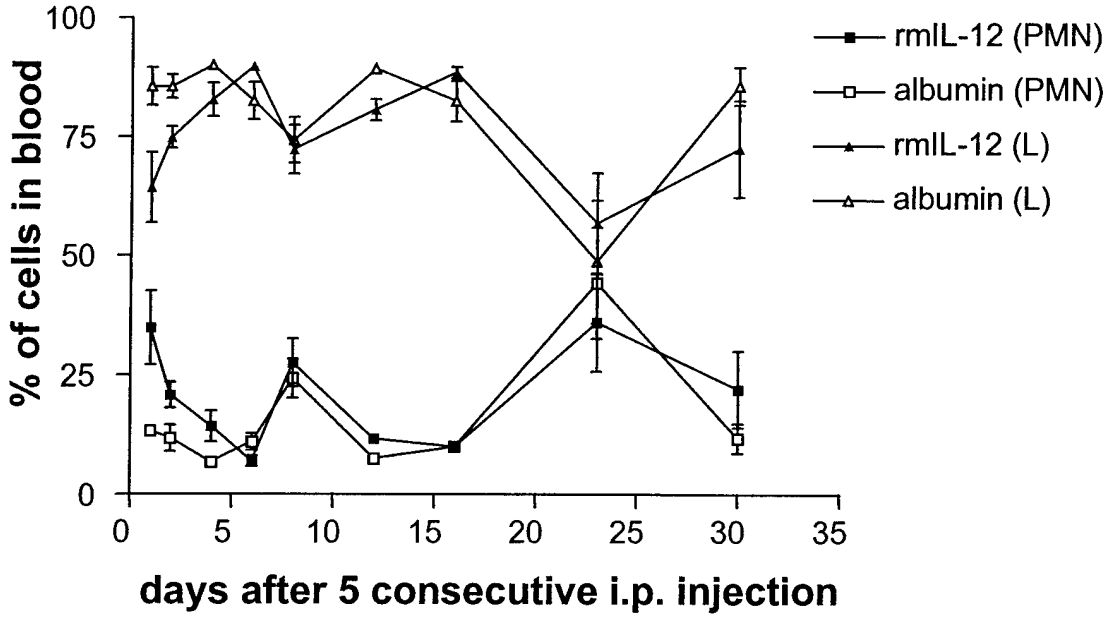
Table 2. Comparison of hematological changes between mIL-12 gene transfer and rmIL-12 administration.

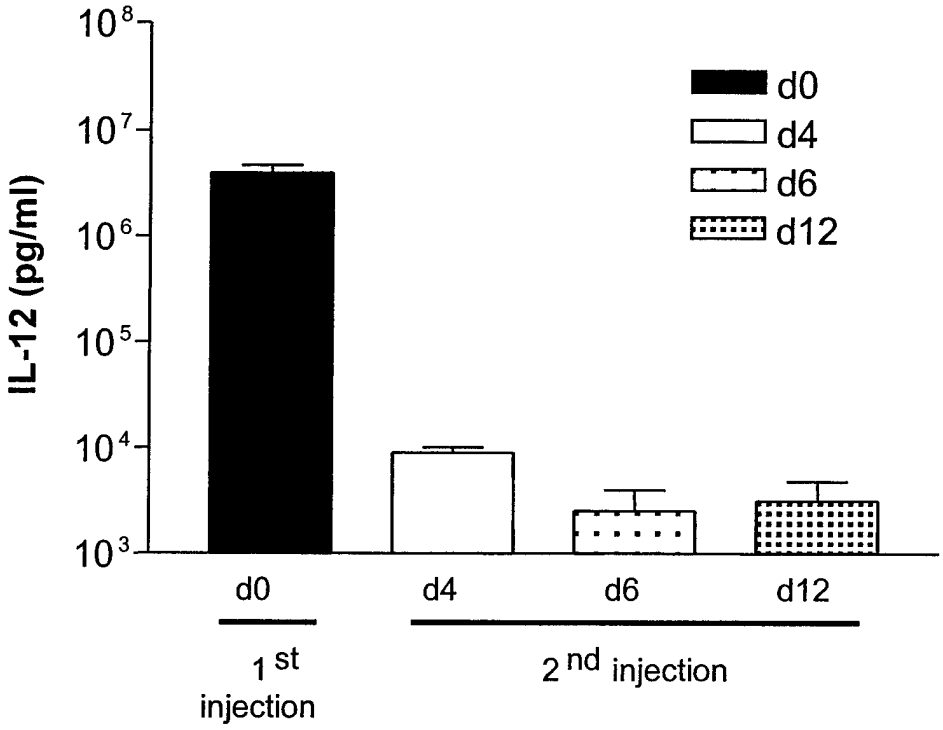
Blood parameter	Treatment	Mean % change compared with untreated at d1								
		d1	d2	d4	d6	d8	d12	d16	d23	d30
Hematocrit (%)	pNGVL3-mIL12	-3.8	-16.2	-48.4	-45.1	-47.9	-23.4	-6.3	-9.5	-8.3
	pNGVL-1	-0.4	-15.3	-10.5	-1.7	-1.8	2.1	3.0	-5.3	-10.8
	rmIL-12	-45.3	-43.9	-30.5	-17.1	5.8	-7.0	-7.2	8.3	6.9
	Albumin	5.6	-1.8	4.4	-2.9	9.5	24.8	0.8	12.4	3.2
Hemoglobin (g/ml)	pNGVL3-mIL12	17.8	0.5	-14.5	-47.0	-60.9	-33.5	-14.8	27.2	1.6
	pNGVL-1	-9.2	-4.4	1.5	11.0	2.6	16.0	-8.4	-16.3	-37.8
	rmIL-12	-57.0	-60.5	-54.5	-42.1	-31.3	26.5	2.9	2.9	-19.8
	Albumin	-1.6	11.8	7.8	6.6	2.5	-2.1	-21.4	-21.3	-40.2

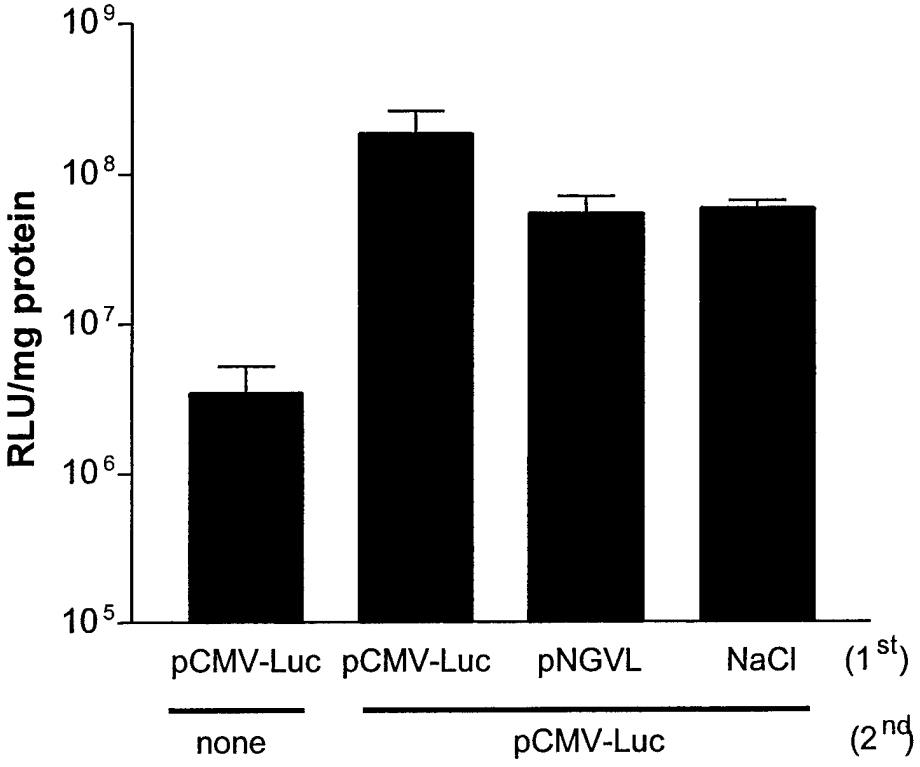
A

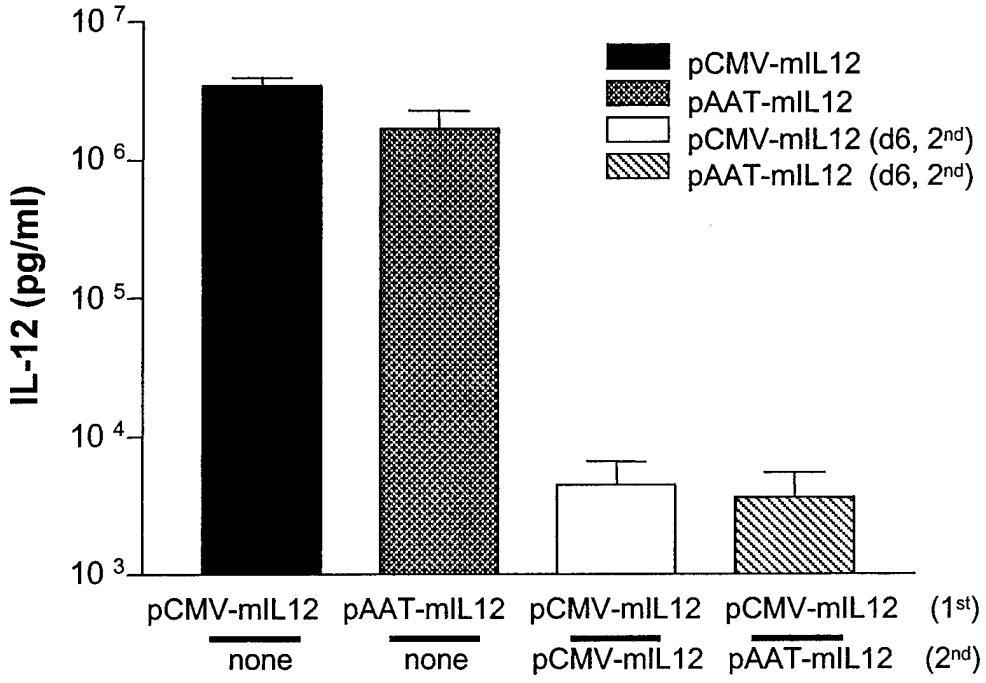

100
75
50
25
0
% of cells in blood
0
5
10
15
20
25
30
35
days after injection
CMV-mIL12 (PMN)
NGVL-1 (PMN)
CMV-mIL12 (L)
NGVL-1 (L)

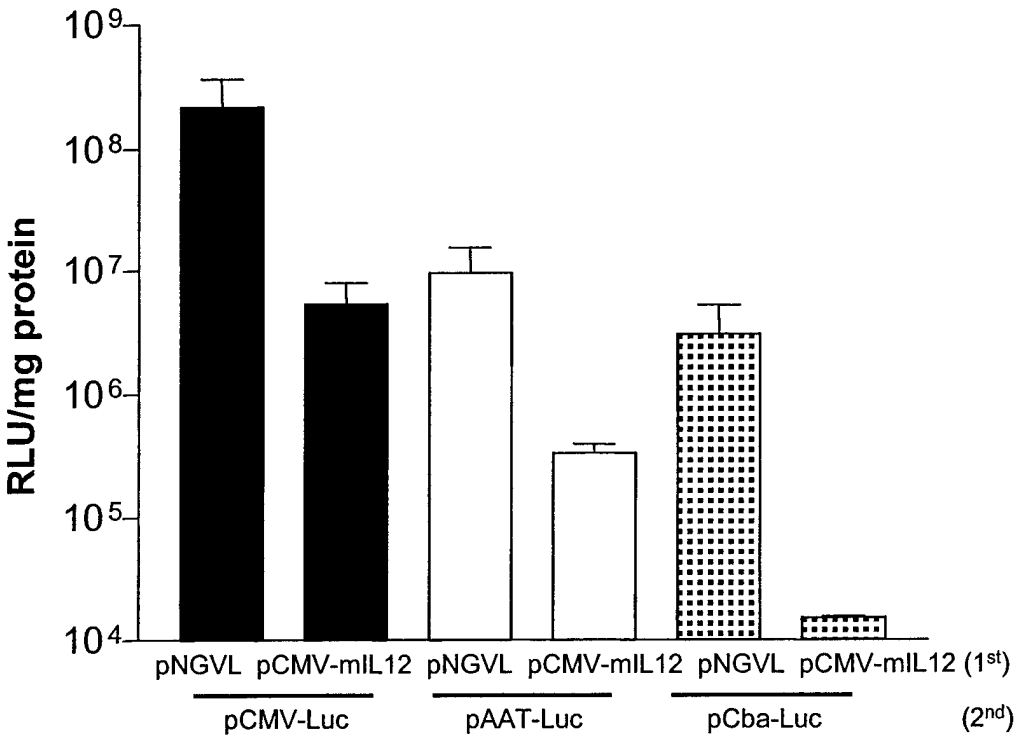
B


100
75
50
25
0
% of cells in blood
0
5
10
15
20
25
30
35
days after 5 consecutive i.p. injection
rmIL-12 (PMN)
albumin (PMN)
rmIL-12 (L)
albumin (L)


IL-12 (pg/ml)
10^8
10^7
10^6
10^5
10^4
10^3
d0
d4
d6
d12
d0
d4
d6
d12
1 st injection
2 nd injection


RLU/mg protein
10^9
10^8
10^7
10^6
10^5
pCMV-Luc
pCMV-Luc
pNGVL
NaCl
(1st)
none
pCMV-Luc
(2nd)


IL-12 (pg/ml)
10 7
10 6
10 5
10 4
10 3
pCMV-mIL12
pAAT-mIL12
pCMV-mIL12 (d6, 2nd)
pAAT-mIL12 (d6, 2nd)
pCMV-mIL12
pAAT-mIL12
pCMV-mIL12
pCMV-mIL12
(1st)
none
none
pCMV-mIL12
pAAT-mIL12
(2nd)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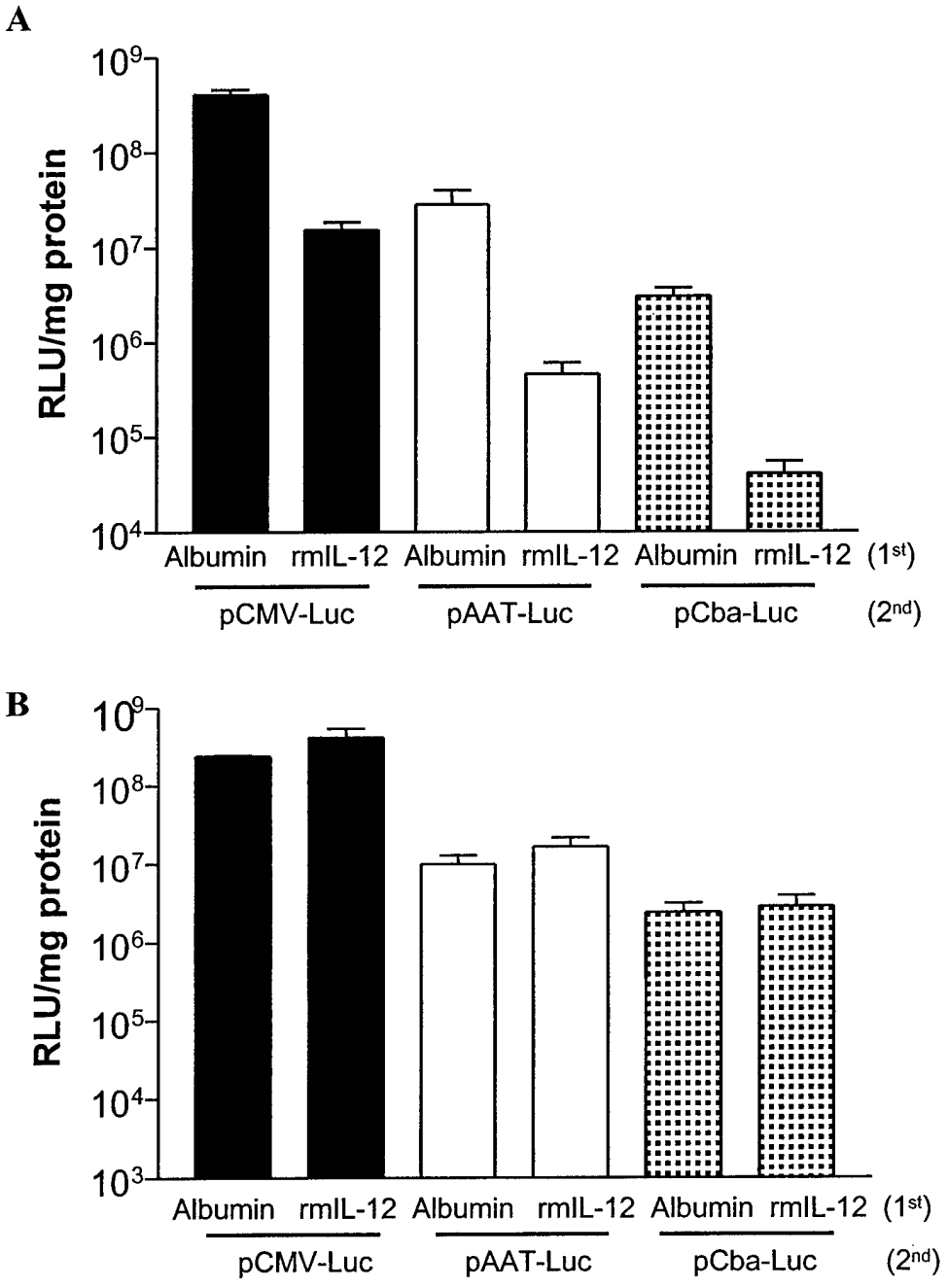
10^8
10^7
10^6
10^5
10^4
pNGVL
pCMV-mIL12
pNGVL
pCMV-mIL12
pNGVL
pCMV-mIL12
(1st)
pCMV-Luc
pAAT-Luc
pCba-Luc
(2nd)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RLU/mg protein
10^9
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10^7
10^6
10^5
10^4
Albumin
rmIL-12
Albumin
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Albumin
rmIL-12
(1st)
pCMV-Luc
pAAT-Luc
pCba-Luc
(2nd)
B
RLU/mg protein
10^9
10^8
10^7
10^6
10^5
10^4
10^3
Albumin
rmIL-12
Albumin
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Albumin
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(1st)
pCMV-Luc
pAAT-Luc
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(2nd)

MUC1 MUCIN AS A TARGET FOR IMMUNOTHERAPY OF CANCER

MUC1 based immunotherapeutic strategies

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1. INTRODUCTION

It has been more than a 100 years since the first attempts of cancer immunotherapy were made based on the assumption that tumor cells are recognized as foreign by the immune system. Over the last decade, there has been a considerable increase in our understanding of immune responses against cancer and the antigenic structures on tumor cells that are recognized by the immune system. Tumor antigens have been classified into distinct categories: tissue-specific differentiation antigens, tumor-specific unique antigens and tumor-specific shared antigens. MUC1 belongs to both the first and the last category.. Although MUC1 is expressed on both normal tissue as well as tumors, it has been extensively studied as a tumor antigen in both basic science as well as applied research for a number of exciting reasons. Some of these include its interesting protein structure, extensive glycosylation (which is altered in tumors) increased expression on tumors and changes in cellular distribution upon malignant transformation. In addition, it is very extensively expressed on a variety of human ductal adenocarcinomas. All these facts justify its use as a candidate for tumor-specific immunotherapy. The object of this chapter is to highlight current and past data concerning experimental and clinical immunotherapy of cancer using MUC1 as the target tumor-rejection antigen.

2. THE MUCIN FAMILY

Mucins are typically large membrane bound or secreted glycoproteins. They are produced by epithelial cells of the gastrointestinal, respiratory and reproductive tracts. To date the mucin family includes as many as 9 different mucins (Allen, 1981, Gendler, 1990, Gum, 1992, Neutra, 1987) the gene for MUC1 being the first to be isolated and sequenced (Gendler, 1990).

Structurally, mucins consist of a core protein moiety (apomucin) that has numerous heavily branched oligosaccharide chains attached to serines and threonine

residues via α1-3-O-glycosidic bonds. O-linked glycosylation of mucin molecules can be so extensive that the sugars could account for as much as 80% of the mass of the molecules. The protein backbones consist of numerous stretches of a tandemly repeated peptide sequence varying in length for different mucins. There is very little sequence homology in the core protein domain among the many members of the mucin family. Hence, the repeats may vary in length and sequence but they all have the serine and threonine residues necessary for O-glycosylation (Griffiths, 1990, Gum, 1995, Swallow, 1987). In addition, the mucin tandem repeats also have one or more proline residues that are thought to determine the specificity of the galactosamine transferase that initiates mucin oligosaccharide synthesis. The mucins do share partial homology in the N- and C-terminal domains (Briand, 1981, Hanover, 1980).

Although the mucin family is large we have focused this chapter on MUC1 for several reasons. Unlike the other mucins which have a more restricted expression primarily on normal epithelia, MUC1 has restricted expression on normal epithelia but is also overexpressed on most epithelial adenocarcinomas derived from different anatomic sites. In addition, as will be described later in the chapter, there are certain characteristics of MUC1 expression on tumors that qualify it as a tumor specific antigen. Hence, most of the immunotherapeutic approaches to date have targeted MUC1 on epithelial adenocarcinomas.

3. EXPRESSION AND FUNCTION OF MUC1 MUCIN

In the past, the product of the MUC1 gene has been assigned several different names including episialin, polymorphic epithelial mucin (PEM), PAS-O, human milk fat globule (HMFG) antigen, DF3 antigen. For the sake of clarity and in keeping with the Human Genome Mapping conventions, we will refer to the MUC1 gene product as MUC1.

MUC1 is expressed by epithelial cells of the breast, lung, colon, pancreas, ovary, uterus, stomach and gallbladder. It is the only member of the mucin family that is a transmembrane glycoprotein (all the others are secreted) (Seregni, 1997, Shankar, 1997). On normal ductal epithelia, MUC1 expression is polarized; i.e. it is found only on the apical surface facing into the lumen of the duct (Fig. 1)(Gendler, 1990). In the adult breast, MUC1 expression increases during pregnancy and lactation and is shed into the milk (Braga, 1992).

MUC1 shares some basic functions with other mucin family members. These include protecting ductal epithelial cells from harsh environments caused by digestive enzymes or preventing colonization by microorganisms. In addition, MUC1 has been implicated in promoting tumor metastasis, in signal transduction and as a ligand for P- and E selectins. In mice, MUC1 expression coincides with ductal lumen formation and it is thought that MUC1 may play a role in duct formation during organogenesis in the developing embryo (Braga, 1992, Hilkens, 1992, Hilkens, 1995, Lesuffleur, 1994). MUC1 knockout mice, however, have shown no abnormalities in duct development (Kardon, 1999) suggesting either no role for MUC1 in this process, or considerable redundancy of molecules (perhaps other mucins) capable of this function.

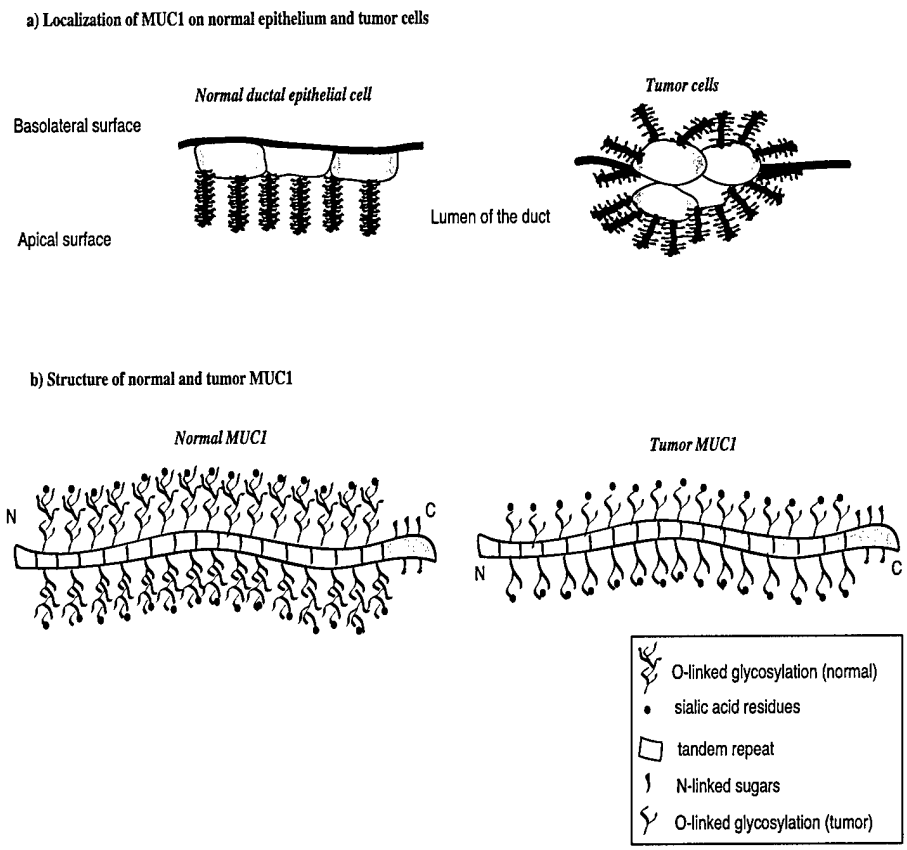

Figure 1. Schematic representation of a) Localization of MUC1 on normal epithelium and tumor cells. MUC1 on normal cells is expressed on the apical surface facing into the lumen of the duct while on tumor cells it loses its polarized expression and is expressed all over the cell. There is also an increase in the level of MUC1 expression on tumor cells. b) Structure of normal and tumor MUC1. Normal MUC1 has highly branched O-linked sugars attached to the serine and threonine residues within each tandem repeat that are terminally sialylated. On tumors, MUC1-associated O-linked sugars lack the highly branched sugar chains due to early termination of O-linked glycosylation but have terminal sialic acid residues. The figure does not show glycosylation of the five potential O-glycosylation sites within each tandem repeat region.

4. STRUCTURE OF MUC1 ON NORMAL AND CANCER CELLS

4.1. Domains of the MUC1 Molecule

The MUC1 molecule can be divided into three regions: a large extracellular domain that makes up most of the molecule, a hydrophobic transmembrane domain and a short cytoplasmic domain. A major portion of the extracellular domain protein backbone consists of tandemly repeated sequence PDTRPAPGSTAPPAHGVTSA, 20 amino

acids in length, rich in serine (10%), threonine (15%) and proline (25%). The serine and threonine residues serve as potential O-glycosylation sites (Gendler, 1995, Lancaster, 1990). As a result of allelic polymorphism, the number of tandem repeats can vary anywhere between as few as 20 and as many as over 120 per allele. Thus, the length of the extracellular domain may vary greatly and in the case of the longer alleles it may extend as much as 400-500nm above the cell membrane. NMR and CD spectroscopy studies have revealed that the proline rich 20 amino acid repeated sequence forms a stable type II β-turn. At 20 amino acid intervals, there are protruding "knobs" with the PDTRP sequence on the tip of each "knob". The stability of the "knobs" and of the overall 3-D molecular structure in solution is highly influenced by the multiplicity of the repeats and degree of glycosylation (Fontenot, 1995, Fontenot, 1993).

A unique sequence of 227 residues proximal to the transmembrane domain includes five N-glycosylation sites and a proteolytic cleavage site that is used during the intracellular processing of the molecule. Even though the protein is cut at that site, the two parts remain associated on the cell surface. C-terminal to this region is a 31 amino acid long hydrophobic transmembrane domain and a 69 amino acid long cytoplasmic domain (Gendler, 1995, Miles, 1999). It has been shown that there are at least two discrete sequence motifs within the MUC1 molecule that specify membrane localization: one in the extracellular domain that confers apical localization and the second at the junction of the cytoplasmic and transmembrane domains that is necessary for specifying surface expression (Pemberton, 1996).

Although the major form of MUC1 is the transmembrane molecule with numerous tandem repeats, other minor forms of MUC1 do exist but their function on either normal or transformed cells remains unknown. These minor forms include secreted MUC1 and MUC1 lacking tandem repeats (Baruch, 1997).

4.2. Glycosylation of MUC1

MUC1 expressed on normal epithelial cells is a heavily glycosylated molecule with highly branched O-linked oligosaccharide chains. Within the 20 amino acid sequence PDTRPAPGSTAPPAHGVTSA, there are five potential O-glycosylation sites, namely the three threonine and two serine residues. During O-glycosylation, N-acetylgalactosamine (Gal-Nac) is linked to serine or threonine residues, followed by the addition of galactose (Gal). The addition of Gal-Nac occurs in a sequential manner first on the two threonines and then the serine (Stadie, 1995). The enzyme β1-6-N-acetylglucosamine transferase then catalyzes the transfer of N-acetylglucosamine (GlcNAc) to GalNAc to form the basic core structure. In vitro glycosylation studies using MUC1 tandem repeat peptides and lysates from human breast or pancreatic tumor cell lines have shown that not all the threonine and serine residues within the tandem repeat region are glycosylated. In fact, in these studies, the threonine in the immunodominant PDTRP epitope was not glycosylated. In addition, these studies suggested that the ability of peptide residues to serve as acceptor substrates is affected by the primary amino acid sequence and by the proximity of the residue to the amino or carboxyl terminus of the peptide substrate (Nishimori, 1994, Nishimori, 1994, Stadie, 1995). In another study, Muller et al., (1997) demonstrated that all five

potential O-glycosylation sites within the tandem repeat region are glycosylated in purified milk MUC1. The discrepancies between these two studies might be reflective of the in vitro versus in vivo systems used to detect glycosylation patterns. The basic core structure of the MUC1 molecule is further extended to form long branched polysaccharides with relatively low concentrations of sialic acid. In addition to the O-glycosylation sites within the tandem repeat, there are five N-glycosylation sites proximal to the tandem repeat region within the extracellular domain that are all that are all glycosylated (Fig. 1) (Miles, 1999, Xing, 1992).

The process of glycosylation followed by sialylation is not completed during the initial transit of newly synthesized MUC1 molecules from the ER to the plasma membrane. To generate extensively glycosylated MUC1, the molecules that reach the cell surface from the ER are repeatedly internalized and recycled through the trans-Golgi network (at least 10 times) before they are released to the outside environment as fully mature (Litvinov, 1993, Pimental, 1996)

4.3. Effect of malignant transformation on MUC1 expression and processing

Although the sequence of the protein backbone of MUC1 remains unchanged upon malignant transformation, there are dramatic changes that occur on the mature molecule that affect its expression pattern and glycosylation status. In adenocarcinomas, MUC1 expression on the cell surface is no longer polarized. There is also a dramatic increase in the number of MUC1 molecules produced and expressed on the surface, that can be as high as 50-fold over normal cell levels. In addition, tumor MUC1 is severely underglycosylated (Fig. 1). Indirect evidence to support the aberrant glycosylation of tumor MUC1 came from studies wherein the monoclonal antibody SM3 and several other MUC1-specific antibodies recognized cancer-associated MUC1 but not the normally processed glycoform secreted into the soluble fraction of milk during lactation (Gendler, 1990). Direct evidence was provided by studies in which Hanisch et al., (1996) analyzed and compared the different MUC1 glycoforms secreted by mammary carcinoma cells (T47D) and MUC1 from a primary ductal breast carcinoma to MUC1 from human milk. Using isopycnic density gradient centrifugation they were able to demonstrate that the major glycans from tumor cell lines and from primary tumor consisted of sialylated, core type glycans that lack L-fucose, a characteristic component of peripheral oligosaccharide regions. The major glycans of tumor MUC1 ranged between trisaccharides to tetrasaccharides. Thus, there appeared to be a premature truncation of O-linked glycosylation. This aberrant glycosylation is considered to be due in part to the increased production of MUC1 requiring increased function of the glycosylation machinery, as well as to known changes in the glycosylation machinery within tumor cells. The MUC1-type O-glycan structures are greatly influenced by the expression and golgi localization of the glycosyltransferases that compete for common substrates. Thus, when the competing enzymes are located within the same golgi compartment, it is the relative activities of these enzymes that determine the glycan structure. Specifically it has been shown that there is about a 10-fold increase in the activity of α2-3-sialyl transferase and a decrease in the activity of β1-6GlcNAc- transferase in some breast cancer cell lines (Brockhausen, 1995). Changes in the activity of these enzymes lead to the premature

addition of sialic acid onto the Gal residues during O-glycosylation thereby preventing further elongation of the carbohydrate side chains. The early termination of carbohydrate elongation results in the accumulation of tumor specific forms of MUC1 that bear short carbohydrates such as 3GalNAcα1-O-Ser/Thr (called Tn), Galβ1 (3GalNAcα1)-O-Ser/Thr (called T) and their sialylated forms sTn and sT (Brockhausen, 1995).

5. IMMUNE RECOGNITION OF MUC1

For a tissue-specific or a differentiation antigen to be considered a tumor-specific antigen there must be changes in either the expression pattern or structure of the molecule on tumors that make it unique and tumor specific. Changes in the MUC1 molecule that occur upon malignant transformation of MUC1-expressing tissues are indeed noticed by the immune system. Early truncation of the oligosaccharide side chains on tumor MUC1 leads to the exposure of antigenic epitopes on the protein backbone that are recognized by B and T cells. Also, changes in the expression pattern such as its overexpression and the loss of polarization results in tumor MUC1 being abundantly expressed over the entire surface of the tumor cell. Thus the molecule is no longer sequestered within the lumen of the duct and is accessible to the effector mechanisms of the immune response. These features of tumor-associated MUC1 make it different enough from normal MUC1 such that B and T cells that recognize tumor-associated MUC1 do not recognize the fully glycosylated protein on normal ductal epithelium.

In this section of the chapter, we will describe the types of MUC1-specific immune responses that can be detected in cancer patients as well as responses that can be primed in vitro using peripheral blood lymphocytes (PBL) from cancer-free individuals.

5.1. Humoral responses to MUC1

Studies that we initially performed on sera from breast, colon and pancreatic cancer patients showed that MUC1-specific antibodies could be detected in approximately 10% of patients. We also showed that these antibodies recognized the PDTRP epitope which is at the tip of the 'immunodominant knob' present within each tandem repeat of underglycosylated tumor MUC1 molecules (Kotera, 1994). More sensitive ELISA assays we have developed since have detected the presence of these antibodies in most patients. Isotype analysis of the anti-MUC1 antibodies in patient serum revealed that such antibodies are primarily of the IgM isotype which is indicative of a helper T cell-independent response In fact, a majority of the antibodies to human MUC1 that have been raised in the mouse, recognize this peptide sequence confirming the immunodominance of this epitope (Xing, 1989). Studies performed by others (Petrarca, 1999, Richards, 1998, Rughetti, 1993) have confirmed the presence of anti-MUC1 IgM antibodies in serum from patients with epithelial tumors such as breast, ovarian, and stomach.

The significance of an ongoing humoral response to MUC1 in cancer patients has not been fully determined. It is tempting to consider these responses potentially therapeutic in light of the expected effector functions of anti-tumor antibodies (such as direct killing of tumor cells by complement fixation, opsonization of soluble antigen and antibody-mediated cellular cytotoxicity, ADCC). In fact, circulating immune complexes of antibodies and MUC1 have been measured in sera from patients with breast cancer and have been correlated with a better clinical outcome (Gourevitch, 1995, von Mensdorff-Pouilly, 1996). These types of observations suggest that it may be desirable to design vaccine and other immunotherapeutic approaches to elicit or amplify MUC1 specific antibodies.

Other lines of investigation that suggest potential anti-tumor effect of the humoral response are studies that have used passive antibody therapy to target tumors expressing MUC1. Radioimmunotherapy using yttrium-90 or I-131 labeled monoclonal antibodies was tested in a phase I clinical trial to treat patients with incurable metastatic breast cancer who had failed standard therapies. Therapy usually resulted in subacute or minimal acute toxicity. Approximately half the number of patients had transient clinically measurable tumor responses that lasted for upto five months post-therapy. One common problem associated with this type of therapy is that patients develop high human anti-mouse antibody titers that interfere with multicycle therapy. One way to overcome this problem is to use a "humanized" antibody (Denardo, 1997, Denardo, 1997).

A radiolabelled antibody has also been tested in a phase I/II study in ovarian cancer patients following surgery and chemotherapy (Hird, 1993). In this study, the monoclonal antibody HMFG-1 (which recognizes a peptide epitope within the tandem repeat region of MUC1) was radiolabelled with yttrium-90 and administered to patients intraperitoneally. No major toxicity was associated with this vaccine. On the basis of survival data from this study, it was concluded that there might be some benefit to using this type of adjuvant therapy in patients with advanced ovarian cancer. Another immunotherapeutic approach is based on bispecific antibodies (BsAbs) which redirect and trigger effector cells to kill tumors. Such antibodies have dual specificities, one for the tumor antigen and the other for the immune effector cell. Katayose et al.(1996) synthesized and tested two such bispecific antibodies: MUC1xCD3 BsAb and MUC1xCD28 BsAb. In vitro experiments confirmed enhanced cytotoxicity of MUC1 expressing tumors by LAK cells. Infusion of both BsAbs followed by injection of in vitro activated LAK cells resulted in delayed tumor growth in a bile duct carcinoma (BDC)-grafted SCID mouse model.

5.2. Cytotoxic T cell Responses to MUC1

In the late 1980s, it was generally accepted that T cells expressing the $\alpha\beta$-T cell receptor (TCR) recognize antigens that are processed and presented as peptide fragments in the groove of MHC molecules. Moreover, this recognition is restricted to targets that are of the same MHC haplotype as the T cell. Antigen recognition appeared to be very different in the case of cytotoxic T cells that recognized MUC1 on tumor cells. We were able to generate a cytotoxic line from the lymph node of a pancreatic cancer patient and showed that this line could recognize and kill pancreatic

and breast-tumor cell lines expressing MUC1 (Jerome, 1991). This killing was inhibited by the MUC1-specific mAb SM-3 (that recognized the PDTRP sequence), by antibodies to the TCR-CD3 complex but not by anti-MHC class I antibodies. This suggested that the native MUC1 molecule was the antigen being recognized by the TCR in a MHC-unrestricted manner. Moreover, the epitope recognized by the CTL line was the PDTRP sequence (later shown in structural studies to be at the tip of the 'immunodominant knob'). These observations were supported by studies wherein CTL lines established using autologous or allogeneic mucin-transfected B cells killed autologous mucin-transfected B cells demonstrating that mucin, and not an alloantigen, was being recognized (Jerome, 1993). Moreover, in order for the MUC1-transfected B cells to be recognized, they had to be treated with a competitive inhibitor of O-linked glycosylation, phenyl-GalNAc presumably to expose the PDTRP epitope.

To explain the phenomenon of MHC-unrestricted recognition of MUC1, it was proposed that the TCRs of a single T cell could interact with multiple epitopes repeated along the extracellular domain of a single MUC1 molecule. Such high avidity interactions could potentially result in cross-linking of the TCR leading to T cell activation. Further investigation into this MHC-unrestricted recognition of MUC1 (Magarian-Blander, 1998) revealed that MUC1 conjugated to microspheres could induce a partial T cell signal accompanied by a transient influx of Ca^{2+} but no translocation of the nuclear factor of activated T cells (NF-AT) into the nucleus or CTL proliferation. Direct recognition of MUC1 on tumor cells however, in the presence of certain accessory molecules resulted in full activation (leading to NF-AT translocation and CTL proliferation). This suggested that full T cell activation of MUC1-specific MHC unrestricted CTL requires additional intercellular interactions such as ICAM-1/LFA-1, and LFA-3/CD2

MHC-restricted responses to MUC1 have also been observed. In fact, peptide STAPPAHGV from the tandem repeat of MUC1 was shown to bind to HLA-1, -A2.1, -A3 and –A11. Secondary responses to this peptide could be detected in lymph nodes from an HLA-A11 breast cancer patient (Domenech, 1995). In addition, it is possible to generate CTL specific for this peptide from healthy HLA-A11 or –A1 positive donors (Domenech, 1995, Hiltbold, 1999). More recently, it has been shown that a peptide from the leader sequence of MUC1 can be used to prime MUC1-specific CTL from HLA-A2 positive healthy donors in vitro (Brossart, 1999). The MUC1 epitopes that are recognized in an MHC restricted manner are not tumor specific since they are not mutated in tumors and can potentially be presented by MHC-class I molecules on the normal epithelial in addition to tumor cells. Hence, although it is important to keep such epitopes in mind as potential targets for anti-tumor responses during immunotherapy, their utility is limited by the possibility that these responses may also target normal ductal epithelia.

5.3. Helper T cell Responses to MUC1

To date, there have been no reports of MUC1-specific T helper cell responses in cancer patients. The lack of detectable T helper responses in cancer patients is surprising considering the high concentration of MUC1 that is detectable in the soluble form in sera and ascites from patients. This soluble form of MUC1 is much more glycosylated

and sialilated while the underglycosylated MUC1 remains tumor associated. In our recently reported studies we have put forth a model to explain the lack of MUC1-specific T helper cell responses. We found that it was possible to prime (in vitro) class II restricted responses to MUC1 using only the unglycosylated form of MUC1 (Hiltbold, 1998). This form of MUC1 was very efficiently processed and presented by antigen presenting cells (APCs) such as dendritic cells (DC) (Hiltbold et al., submitted). Interestingly, using the fully glycosylated form of MUC1 (purified from patient ascites) we were unable to prime MUC1-specific MHC class II restricted responses. We found that this fully glycosylated form could be taken up by DC but remained in the early endocytic compartments and was not trafficked further into the cell to be processed and presented in the context of MHC class II molecules. Thus we believe that the lack of a detectable helper cell response in cancer patients could be explained by the inability of APCs such as DC to process the soluble form of MUC1 antigen to the immune system. This result is very significant because it also rules out potential tolerance of the helper T cell compartment to MUC1. It is obvious that supplying the underglycosylated form of the MUC1 antigen in a vaccine preparation would be expected to elicit a helper T cell response which in turn may allow expansion of both the humoral and the cytotoxic T cell response against this antigen.

6. TARGETING MUC1 WITH IMMUNE EFFECTOR MECHANISMS

Immune responses against MUC1 that are observed in cancer patients are a testament to the immunogenicity of this molecule. However, these existing responses are limited in their repertoire (IgM and CTL, no other IgG isotypes and no helper cells) as well as intensity (low antibody titers, low CTL frequency) and incapable of irradicating tumors in these individuals. Over the past 10 years there have been numerous attempts to enhance the existing responses or induce new immune responses against MUC1 by incorporating this antigen into various vaccine formulations. A number of studies have been carried out in animal models and in phase-I trials in humans. Although in various animals models MUC1-based vaccines have elicited a range of immune responses and in some cases protection from tumor growth, what remains a challenge is to augment MUC1-specific immune responses in cancer patients to irradicate tumors without causing massive destruction of normal tissues that express MUC1.

6.1. Animal Models for Testing MUC1 Vaccines

Numerous studies testing vaccine formulations containing human MUC1 have been performed in conventional mice. Although this model system has been useful for determining the repertoire of MUC1-specific immune responses that could be induced, it has not been an ideal preclinical model that could predict the type of an immune response that could be expected from a cancer patient who not only might still harbor a considerable number of cancer cells, but also have MUC1 expressed on normal tissue. Human MUC1 shares very little homology to mouse MUC1 and is therefore highly immunogenic in conventional mice. How immunogenic human MUC1 can be in humans cannot be predicted from this animal model. A more relevant mouse model

that has been developed is the MUC1 transgenic mouse model wherein human MUC1 is a self-antigen with an expression pattern similar to that seen in human tissues (Peat, 1992, Rowse, 1998). In this model that is currently under study by several groups (Gong, 1998, Gong, 1997, Rowse, 1998, Tempero, 1999, Tempero, 1998) it is possible to explore issues related to MUC1 immunogenicity, tolerance or autoimmunity, all of which could profoundly affect the choice of immunotherapy.

The chimpanzee has been a very useful animal model in which to test MUC1-based vaccines. Since chimpanzee MUC1 and human MUC1 are considered to be identical, any responses induced in this model would be expected to be induced in humans as well. Several vaccines have been tested in this model with very encouraging results. The limitations to this model however are the cost and availability of animals and the lack of transplantable tumors in the chimpanzee. Hence, while the questions of immunogenicity, tolerance and autoimmunity issues are appropriately addressed, the effect of immune responses elicited by various vaccinations on tumor rejection cannot be evaluated.

6.2. Clinical Trials in Cancer Patients

Several phase-I clinical trials of MUC-based vaccines have been carried out over the past few years all over the world. Phase I trials are primarily carried out to test the potential toxicity of a vaccine preparation. The immune responses are evaluated only as a secondary aim. One common problem associated with phase I clinical trials is that the patients participating in these trials most often have late stage disease or have received previous therapies such as chemotherapy or radiation therapy and are often severely immunocompromised. Hence, it is very difficult to evaluate the true immunogenecity of the vaccine formulations tested. More recently, trials have been initiated in patients with early disease who are less immunocompromised. The true potential of cancer vaccines, however, including MUC1 based vaccines, will only be revealed when it becomes possible to use these in healthy young individuals at risk for development of cancer. The immune system has been harnessed through vaccinations to protect from infectious diseases, and should be able to respond the same way in cancer prevention.

6.3. MUC1 Peptide-based Vaccines

Use of synthetic peptides in vaccine formulations to induce either humoral or cell mediated immunity requires considerable understanding of antigenic epitopes that can be recognized by either T cells or B cells. Since B cells recognize antigen in its native form, B cell epitopes must be accessible on the soluble protein or on the surface of the tumor cell. B cell epitopes can be either a linear sequence or composed of amino acids from different parts of the molecule yielding a conformational epitope. In the case of MUC1, studies have identified the PDTRP sequence within each tandem repeat to be the immunodominant tumor-specific B cell linear epitope. However, its binding to the antibody receptors is dependent not only on the linear sequence but also on the 3D structure that this sequence assumes in the context of the longer amino acid sequence of the underglycosylated tandem repeat on tumor MUC1.

Since CTL play an important role in recognizing and killing tumor cells, it is imperative for anti-tumor peptide vaccines to include epitopes that can be recognized by CTL. These epitopes are by and large short peptides (8, 9 or 10 amino acids in length) that can bind one or more human HLA Class I molecules. As mentioned before, several of such epitopes have been identified in the MUC1 sequence. In addition, and in our opinion most important, peptide vaccine must include one or more helper T-cell epitopes. These are longer peptides (12-20 amino acids in length) that bind to human HLA Class II molecules. Elicitation of a strong humoral and cell-mediated immunity requires T cell help, as does the establishment of tumor-specific memory responses. As we will discuss below, tumor-specific helper epitopes are often substituted in vaccines with heterologous proteins that can stimulate helper cells. While these may be useful in the generation of a stronger antibody or CTL response at the time of vaccination, the helper cell memory that is established is specific for the heterologous protein and will not be triggered at a later date in the case of tumor recurrence. Thus secondary anti-tumor responses will be severely compromised.

6.3.1. *MUC1 peptides conjugated with heterologous protein carriers*

Ding et al. (1993) tested a vaccine composed of MUC1 synthetic peptides containing the PDTRP epitope conjugated to a protein keyhole lympet hemocyanin (KLH) and administered to mice together with RIBI Detox adjuvant. In addition, they also tested a chimeric peptide containing two tandem repeats of a single MUC1 epitope and a tetanus toxin universal helper T cell epitope. Such immunizations were able to induce strong MUC1-specific delayed type hypersensitivity (DTH) reactions but no CTL responses. They also resulted in delayed tumor development. In a clinical trial carried out in patients with metastatic breast cancer, sixteen patients were immunization with a low dose (5μg) of a 16 amino acid MUC1 peptide conjugated to KLH and administered together with DETOX as an adjuvant. Only three patients developed weak anti-MUC1 IgG responses. In addition, seven out of eleven patients tested for MUC1 specific CTL activity showed the presence of class I restricted MUC1-specific CTL. These were shown to be restricted to HLA-A2, -A1 and A11 (Reddish, 1998).

In other studies using peptide-carrier conjugates, immunizing mice with MUC1 peptide (containing two tandem repeats) linked with diphtheria toxoid (DT) induced a significant DTH reaction and antibody response but no CTL response. This response also resulted in delayed tumor development (Apostolopoulos, 1995). These mouse studies formed the basis for a phase-I clinical trial on 13 patients using MUC1 peptide-DT conjugates as the immunogen administered in increasing doses from 100μg up to 1mg of peptide. Although this trial showed that there was no significant toxicity associated with this form of immunization, the immunogenicity of the vaccine was not very impressive and was similar to what had been observed in the mouse model as far as the types of immune responses that were generated. Only weak antibody and DTH responses were detected in several patients.

In an effort to improve upon these vaccines and induce more cellular responses rather than antibody responses, efforts were made to target MUC1 to receptors on antigen presenting cells (APC) for more efficient antigen uptake and processing. For this, a MUC1 peptide sequence containing five tandem repeats was conjugated to mannan (to target the mannose receptors on APC such as macrophages

and dendritic cells) and was administered to mice intraperitoneally three times at weekly intervals. It was shown that the oxidation state of the immunogen highly influenced the quality of the immune response induced. Under oxidizing conditions (which aids in the formation of Schiffs bases), immunization resulted in cellular responses characterized by high CTL precursor (CTLp) frequency, circulating CD8+ CTLs, a significant DTH response, but little antibody production. This correlated with a Th1-type response (as indicated by IFN-γ production) and significant tumor protection. In contrast, the reduced form of the immunogen induced predominantly antibody responses that had very little tumor protective effect that correlated with IL-4 production (a Th2-type response). (Apostolopoulos, 1996, Apostolopoulos, 1995)). It is not clear why the oxidation status of the antigen affected the type of responses generated. A possible explanation is that processing of the immunogen is affected in some way and hence the oxidized form is targeted to the intracellular processing pathway for presentation in class I while the reduced form follows the endocytic route and is presented in class II. In addition, Apostolopoulos et al., (1998) showed that coadministration of Cyclophosphamide (an immunosuppressive agent) and the oxidized form of the MUC1-mannan conjugate enhanced the CTLp frequency approximately 10-fold (from 1/84,900 without Cyclophosphamide to 1/8,100 with Cyclophosphamide). Surprisingly, in contrast to the murine studies, when cynomologus monkeys were immunized with oxidized MUC1-mannan conjugates, a predominantly humoral response was observed and no detectable CTL response (Vaughan, 1999). Similar observations were made in a clinical trial carried out in 25 patients with metastatic breast, colon, stomach or rectal cancer. Approximately half the number of patients produced significant titers of IgG1 anti-MUC1 antibodies. T cell proliferative responses were seen in 4 out of 15 patients and CTL responses in only two out of 10 patients (Karanikas, 1997).

6.3.2. *MUC1 peptides plus adjuvant*

Another approach to enhancing the immunogenicity of MUC1 in vivo has been to coadminister MUC1 peptide with various adjuvants. These adjuvants activate APC such as macrophages and DCs which in turn enhances their APC function, resulting in increases in humoral and/or cell-mediated immunity. Taking into account the importance of cell-mediated responses in eradicating tumors, most often the adjuvants chosen are those known to skew the immune response towards a Th1 type response. In one of our studies, which was the first ever cancer peptide vaccine to be tested, a phase I clinical trial was carried out using as a 105 amino acid long synthetic MUC1 peptide (slightly longer than 5 tandem repeats) administered with BCG as the adjuvant (Goydos, 1996). Post immunization, the patients were monitored for enhanced MUC1-specific antibody, CTL and DTH responses. No changes in DTH and antibody responses were observed. There was an increase in the number of MUC1-specific CTL post-immunization but they were still too few to be of therapeutic value. This further emphasizes the importance of activating helper T cells in order to expand CTL and enhance CTL function

A study we carried out in the chimpanzee model was aimed at eliciting MUC1-specific cell-mediated immune responses (TH1type). Chimpanzees were immunized with the 100 amino acid synthetic MUC1 peptide admixed with

recombinant LeIF (Leishmania braziliensis homologue of the eukaryotic initiation factor) as an adjuvant. As expected, such a vaccine was able to induce a $CD4^+$ response that was of the Th1 type as well as a CTL response. The animals were monitored for one year post-vaccination and there appeared to be no detectable symptoms of autoimmunity (Barratt-Boyes, 1999).

6.3.3. *MUC1 peptides as particulate antigens*

Poly (d,l-lactic-co-glycolic acid) or PLGA microspheres are made from a biodegradable biocompatible polymer and have only recently gained attention as antigen delivery systems. Their particulate nature contributes to an increased immunogenicity of antigens encapsulated in such microspheres by facilitating phagocytosis by APC. The microspheres loaded with antigen are taken up and either remain in endocytic vesicles or are released into the cytoplasm. The antigen that is released can then be processed and presented either in MHC class I or class II for presentation to both CTL and helper T cells. Newman et al. (1998), used PLGA microspheres containing a 24 amino acid MUC1 peptide (from the tandem repeat region) with or without monophosphoryl lipid A(MPLA) to immunize mice. They found that immunization with MUC1 peptide loaded PLGA microspheres induced IFN- γand no IL-4. Incorporation of MPLA into the MUC1 peptide loaded microspheres not only induced an increase in IFN- γproduction but also in antibody switching to the IgG isotype. Our own experience with a 40 amino acids long MUC1 peptide (two tandem repeats) encapsulated in PLGA microspheres shows that immunization of MUC transgenic mice results in specific immunity that protects from tumor challenge (Soares et al., submitted).

6.3.4. *MUC1 peptides in liposomes*

Another method of enhancing antigen uptake is to use liposomes as the antigen delivery system. Liposomes containing peptide antigens are prepared by mixing the peptides with a suspension of phospholipids under conditions that form vesicles bounded by a lipid bilayer. The liposomes are thought to fuse with the cell membrane of APC thereby releasing their contents into the cytoplasm. Thus, antigens delivered by liposomes are presented primarily in the context of class I MHC. Samuel et al. (1998) tested a MUC1 vaccine formulation in the mouse model for immunogenicity and anti-tumor activity. This vaccine consisted of 24 amino acid MUC1 peptide encapsulated with MPLA in multilamellar liposomes. They showed that the tumor protection mediated by the vaccine correlated with a Th1 type of response (as indicated by IFN- γ production and the production of MUC1-specific IgG2a antibodies).

6.4. MUC1 Carbohydrate Vaccines

As discussed previously, MUC1 on tumors is underglycosylated and new carbohydrate moieties are expressed that serve as tumor antigens. Sialyl-Tn (sTn) is one such disaccharide antigen. Initially, it was thought that carbohydrate immunogens might have certain limitations, such as inability to activate helper T cells. One way to get around the lack of helper T cell activation, has been to conjugate the carbohydrate antigen to a protein carrier. This type of vaccine would be expected to efficiently

activate B cells and help them switch to various Ig isotypes and generate memory B cells. This vaccine would not be expected to induce tumor-specific memory T cells. There are recent reports in the literature that murine T cells can react specifically against carbohydrate antigens including the tumor associated Tn antigen [Galli-Stampino, 1997 #20; (Springer, 1984). This gives carbohydrate antigens a renewed importance as tumor-specific immunogens (Kieber-Emmons, 1999, Lo-Man, 1999).

A vaccine formulation consisting of the Thomsen-Friedenreich (TF) antigen conjugated to KLH administered with RIBI as an adjuvant was tested in the murine system (Henningsson, 1987). The TF antigen is a disaccharide Gal β1-3 GalNAc carbohydrate epitope expressed on tumors. This vaccine not only induced DTH and antibody responses specific for carbohydrate determinants expressed on tumors but also appeared to confer some protection against tumors. Another carbohydrate antigen, sialyl-Tn antigen conjugated to KLH admixed with RIBI adjuvant was administered to mice and metastatic breast cancer patients. IgG responses were detected in both mice and humans, but the responses in humans were much weaker (Longenecker, 1993). This is consistent with the notion that human MUC1 is a foreign antigen in conventional mice and would induce more potent responses than might be expected in MUC1 transgenic mice wherein human MUC1 is a self antigen. In other studies carried out in patients with breast cancer or breast, ovarian and colorectal cancer, a vaccine formulation consisting of sialyl-Tn conjugated to KLH emulsified in DETOX-B SE adjuvant, induced anti-sialyl-Tn IgG responses. There appeared to be a direct correlation between the level of the IgG antibody response and survival suggesting that such IgG antibodies have a therapeutic benefit for the patients. The exact contribution of antibody versus cellular responses towards increased patient survival is unclear since potential cellular immune responses to sTn were not analyzed in these studies (MacLean, 1996, MacLean, 1996). It has also not been convincingly shown that antibodies elicited by these vaccines crossreact with patients tumor cells, thus it is hard to postulate what effector mechanisms are at play in the tumor-specific response in these cases.

More recently, the sTn-KLH vaccine was administered multiple times to seven ovarian and 33 breast high risk stage II/III and stage IV cancer patients following high dose chemotherapy and stem cell rescue (Sandmaier, 1999). Both the cellular and humoral immune responses were studied. As seen in previous studies using this vaccine, most patients developed anti-sTn IgG responses that peaked after the fourth or fifth immunization. Seventeen patients developed proliferative responses to sTn. In addition, antigen specific IFN-γ production was detected in 11 out of 26 patients. An increase in the lytic activity against a sTn^+ cancer target after immunotherapy was also observed. However, the tumor specificity and MHC restriction of this response remains to be determined

6.5. MUC1 DNA Vaccines

6.5.1. *Recombinant Viral Vectors*

Recombinant vaccinia virus containing MUC1 cDNA has been tested as an immunogen in both animal models as well as in humans. Use of such viral vectors to deliver antigens in vivo has certain advantages, the primary one being high levels of

expression of the antigen. In addition, since the virus replicates within the infected cell, the antigen is processed and presented in MHC class I. There have been certain difficulties associated with the use of vaccinia to deliver MUC1. During viral replication, the vaccinia genome is subject to a high degree of homologous recombination that can result in the deletion of most or all MUC1 tandem repeats in which reside most important antigenic epitopes. Nevertheless, vaccinia constructs have been used to immunize mice. One such construct that maintained expression of three to four tandem repeats per molecule when injected into mice induced MUC1 specific antibodies but no detectable CTL. The immunization also caused delayed tumor development (Acres, 1993)

In another study, Akagi et al., (1997), tested in a murine model a recombinant vaccinia viral construct containing the "mini" MUC1 gene containing only ten tandem repeats in order to discourage deletions due to vaccinia recombination. In addition, they also tested an admixture of vaccinia constructs containing either the "mini" MUC1 gene (rV-MUC1) or the gene for murine costimulatory molecule B7-1 (rV-B7-1). Cytolytic responses were detected with both vaccines, however, enhanced responses were seen only with the admixture vaccine. Both vaccines conferred protection against tumor in a tumor prevention model following two administrations. Two administrations of rV-MUC1 were insufficient to confer protection against tumor in a tumor therapy model. But, when mice were primed with rV-MUC1 and rV-B7-1 followed by two administrations of rV-MUC1 there was 100% survival in the tumor therapy model. More recent efforts have been directed at enhancing the immune response to MUC1 by using vaccinia virus constructs co-expressing MUC1 and IL-2. These have been tested both in the mouse model as well as in patients with advanced breast cancer in a phase I clinical trial (Balloul, 1994, Scholl, 1997).

6.5.2. *Naked MUC1 DNA*

Vaccination with naked DNA is a recently developed immunization strategy in which a plasmid coding for the antigen of interest is injected directly into the muscle. This is then taken up and expressed by muscle cells and dendritic cells. Thus, DNA vaccination offers yet another method to target the endogenous processing and presentation pathway. DNA vaccination has two major advantages: it is a method that has been shown to induce both cellular and humoral immunity and, in addition, prolonged expression of the antigen by cells that have taken up the DNA results in the generation of significant immunological memory. It is important to keep in mind that persistent expression of the antigen could also lead to a state of autoimmunity or a state of immunological unresponsiveness. Not enough has been done yet with MUC1 DNA to know the parameters of its immunogenicity.

Graham et al., (1996) investigated the immunostimulatory potential of naked cDNA in the mouse model. Mice were immunized intramuscularly with different doses of MUC1 cDNA and both tumor rejection and immune responses were examined. This immunization protocol resulted in tumor protection that appeared to be dose dependent (the optimal dose being 50-100 μg of DNA). Humoral responses were also seen, however, there was no correlation between tumor protection and the presence of MUC1-specific antibodies. In addition, the immunization alone was not

sufficient to induce detectable MUC1-specific CTL although they were detected only in previously immunized mice post-tumor challenge.

6.6. Cellular MUC1 Vaccines

Priming of an effective cellular immune response requires antigen presentation within MHC class I /II on professional APC high in co-stimulatory and adhesion molecules. Tumors are considered to be poor antigen presenting cells since they are known to express low levels of the costimulatory and adhesion molecules necessary for T cell activation (Chen, 1993). Since antigen presenting cells like B cells, macrophages and dendritic cells can provide the necessary costimulatory and adhesion signals for T cell activation, they have been used as cellular vaccines designed to present MUC1 in vivo in the chimpanzee and murine model systems.

We carried out the first such study in the chimpanzee model using as a vaccine autologous EBV immortalized B cells transfected with human MUC1 cDNA (Pecher, 1996). The B cells were treated prior to vaccination with a potent inhibitor of O-linked glycosylation in order to expose tumor-associated epitopes on the transfected MUC1. Increases in MUC1-specific CTL precursor frequencies were observed in immunized animals. Moreover, since the response was directed at the underglycosylated form of MUC1, no toxicity associated with autoimmunity was noted presumably because normal tissue expresses the fully glycosylated form of MUC1. In addition, no MUC1-specific antibodies were detected.

In another study, we tested the efficacy of a different cellular vaccine consisting of antigen-loaded peripheral blood derived dendritic cells (generated in vitro with GM-CSF and IL-4) in the chimpanzee model (Barratt-Boyes, 1998). In vitro-generated dendritic cells were pulsed with synthetic MUC1 peptide and then injected intravenously. Following a boost with soluble peptide plus adjuvant, the humoral and cellular responses were analyzed. Antibody responses were detected in one out of four animals but only following the boost. No MUC1-specific proliferative T cell or humoral responses were detected following the single innoculation with peptide-pulsed dendritic cells, although such responses to the other antigen (ovalbumin) used in this system were detected. The results of this study suggest that multiple boosts with peptide-pulsed DC might be necessary to induce humoral and cellular responses to the self antigen MUC1 in the chimpanzee.

A number of different cellular vaccines have been tested in the murine model as well. Initial efforts were directed at using mouse tumor cells transfected with human MUC1 cDNA to immunize mice. In one such study, immunization with MUC1-transfected mouse mammary tumor cells resulted in reduced tumor incidence when mice were challenged with the same tumor (Graham, 1995). In another study, it was shown that immunization with MUC1-transfected murine 3T3-tumor cells was able to induce MUC1-specific CTL activity and confer partial protection against a subsequent challenge with the same tumor.

More recently, the choice of a cellular vaccine has been a dendritic cell modified in some way to present MUC1. We used a retroviral vector MFG-MUC1 containing MUC1 cDNA to transduce an immortalized murine dendritic cell line. This MUC1 expressing DC line was then used to immunize mice and the humoral and cell-

mediated immune responses generated were analyzed. This vaccine not only induced MUC1-specific humoral responses, but also cytolytic and proliferative responses. This was the first MUC1 vaccine that was able to stimulate all three effector arms of the adaptive immune response (Henderson, 1998). In another approach, Gong et al. (1997) tested the immunogenicity of MUC1 when presented on DC-tumor cell hybrids. This strategy is aimed at combining the potency of dendritic cells as antigen presenting cells with the multiple tumor-associated antigens on tumor cells to elicit strong tumor-specific immunity. The efficacy of this approach was measured by the strength of the anti-MUC1 response elicited. As expected, immunization led to the induction of a potent immune responses and prevention of MUC1+ tumor growth. In addition, this vaccine also caused rejection of established metastases. Both $CD4^+$ and $CD8^+$ lymphocytes appeared to be involved.

We have evaluated the potential of a cellular vaccine (DC-based) in direct comparison with previously discussed immunization approaches such as peptide plus adjuvant vaccines. MUC1 transgenic and conventional mice were immunized with 1) 140 amino acid synthetic MUC1 peptide-pulsed in vitro generated dendritic cells, 2) 140 amino acid synthetic MUC1 peptide plus adjuvant (murine GM-CSF, a cytokine previously shown to play an important role in regulating dendritic cell differentiation and function as well as increase T cell effector function), and 3) 140 amino acid synthetic MUC1 peptide coadministered with a potent adjuvant SB-AS2 that contains monophosphoryl lipid A (provided by SmithCline Beecham). As expected the three different approaches induced very different immune responses, the cellular vaccine induced primarily cell-mediated responses while the adjuvant-based vaccines induced humoral responses. In addition, it was seen that the weaker adjuvant GM-CSF induced responses only in conventional mice, while the more potent SB-AS2 induced responses in both the conventional and MUC1 transgenic mice. Tumor rejection studies using a syngeneic MUC1-expressing transplantable tumor revealed that only the peptide-pulsed DC group was capable of rejecting tumor, consistent with the induction of a TH1 type response (Soares and Finn, submitted).

7. CONCLUDING REMARKS

The experimental evidence presented here on MUC1-based immunotherapy illustrates some of the successes of the field of immunotherapy in animal models as well as the challenges we face in translating basic and clinical research into successful cancer therapies. The development and use of animal tumor models, such as of syngeneic tumors, spontaneously arising tumors and human tumor xenografts in immunodeficient mice, have allowed us to evaluate numerous novel anti-cancer therapies. Such studies have contributed to our understanding of the interaction between immunocompetant cells and their products on the one hand and tumor cells on the other as well as the relationship between them. MUC1 is one of the few well characterized tumor antigens. Efforts are currently being directed at discovering new ones. Most vaccination strategies to date have been based on a single tumor antigen. It is conceivable that anti-tumor vaccines consisting of more than one tumor antigen might be more effective at eliciting anti-tumor responses leading to tumor rejection. In addition, it is important to

keep in mind that there are also certain host-related factors that are obstacles to the successful application of cancer vaccines, such as the immunosuppressive tumor microenvironment. Studies are being directed at overcoming such immunosuppression. It is possible that present vaccination strategies being tested will be more effective in patients with smaller tumor burdens. With the increased sensitivities of cancer screening programs, one can look toward a future wherein disease-related abnormalities are detected early in premalignant states and are treated in the clinic with preventive immunotherapeutic vaccines.

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Specific Down-regulation of HER-2/neu Mediated by a Chimeric U6 Hammerhead Ribozyme Results in Growth Inhibition of Human Ovarian Carcinoma

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ABSTRACT

U6 expression system was explored for efficient expression of a ribozyme against a human proto-oncogene c-neu. A hammerhead ribozyme (neuRz) and the control mutant ribozyme (MRz) were targeted to cleave c-neu mRNA at the tyrosine kinase domain. *In vitro* cleavage showed that neuRz was very active while MRz was not. Near maximal target cleavage observed at a low ribozyme: target ratio (0.1) suggests that neuRz has good activity and turnover capability under physiological conditions, i.e, < 5 mM $MgCl_2$ and 37 °C. Chimeric U6 ribozyme was expressed at about 5 x 10^6 copies/ cell in 48 h in an ovarian carcinoma cell line SKOV-3.ip1. Partial down-regulation of c-neu mRNA and protein was observed in a dose-dependent manner in cells transiently transfected with U6neuRz-, but not with MRz-containing plasmid. Sorted transfectant demonstrated dramatic growth inhibition with the neuRz-expressing cells. *In vivo* delivery of naked DNA carrying U6neuRz resulted in specific growth inhibition of a subcutaneous solid tumor of SKOV-3.ip1 in SCID mice. Our results demonstrated that the U6 expression system is very efficient and suitable for the expression of a hammerhead ribozyme. Moreover, specific down-regulation of c-neu results in growth inhibition of ovarian cancer cells *in vitro* and *in vivo*.

Keywords: HER-2/neu Ribozyme, Ovarian Cancer Growth Inhibition, U6 RNA, Gene Therapy

INTRODUCTION

The c-neu proto-oncogene (also known as HER-2/ erbB-2 or NGL) encodes a transmembrane receptor of 185 kD (p185) with intrinsic tyrosine kinase activity, which is important for its intracellular signal transduction (1). Overexpression of c-neu has been frequently associated with gene amplification. Many types of human cancer including breast (20-30 %), ovarian, lung, gastric, colon and oral cancers are found to overexpress p185 (2-7). Moreover, the level of gene expression is associated with the disease stage, lymph node and visceral metastases, relapse, poor prognosis and low survival rate in breast, ovarian and lung cancer patients (3,4).

The cellular function and signaling pathways of c-neu are not fully elucidated. It is believed that c-neu plays an important role in tumorigenesis, DNA repair, drug resistance and metastasis (8,9). Therefore, c-neu has become an important target for tumor biology as well as therapeutic intervention in cancer gene therapy. Many gene therapy strategies have been developed to abrogate c-neu expression in cancer. These include antisense oligonucleotide (10,11), antisense RNA (12), ribozyme, triplex-forming oligonucleotides binding to HER-2 promoter to inhibit transcription (13,14) and c-neu specific transcriptional repressors (E1A, SV large T antigen and PEA3). Owing to the high level of c-neu over-expression and the long half-life of c-neu, successful down-regulation of c-neu seems to require either sustained or high level of gene expression of the therapeutic gene such as antisense RNA or ribozyme expressed from adenoviral vector or stable clones and phosphorothiaote oligonucleotide.

Ribozyme is a catalytic RNA which has the capability of multiple turnover and acts in trans or cis for irreversible cleavage of target RNA (15). Among different types of ribozymes discovered, the hammerhead ribozyme has attracted most of the interest in gene therapy owing to its intrinsic advantages over the others. First, it has a relatively simple structure making the synthesis easy. Second, it has a well-defined and short

catalytic core. Third, the flanking arms of stem I and III can be nearly any sequence so that the ribozyme can be tailor-made to bind and cleave any target RNA with high specificity. Fourth, the short ribozyme can be chemically modified to further improve kinetics of cleavage and stability (16). Many different hammerhead ribozymes targeting to oncogenes (H-ras, N-ras, *bcr-abl* , c-fos, and human papilloma virus E6 and E7) and HIV have been developed with success (15).

In order to discern the specific role of a gene, specific down-regulation of the target gene becomes particularly important and essential. Intracellular expression of ribozyme seems to be a more defined alternative to fulfill this criterion compared to antisense. It is now known that phenotypic changes resulted from antisense oligonucleotide treatment may not be related to specific inhibition of the target gene (16). Chemical modifications of phosphodiester oligonucleotide, such as phosphorothioate ODNs, causes non-specific inhibition of translation machinery, shutting down of normal cellular processes like receptor internalization and replacement and results in non-specific toxicity (11,16). On the other hand, a ribozyme-expression system is more useful for the study of specific gene function. It is because simple comparison of the effect between an active ribozyme and the corresponding inactive ribozyme control will reveal more accurately the function of the target gene.

Development of efficient expression system for the expression of a small piece of RNA in the cell is a major challenge in non-viral gene therapy. It is because most of the mammalian expression systems employ powerful viral promoters and these viral promoters are not very efficient and specialized for the expression of small RNA. We previously showed that expression of a small antisense RNA from the CMV promoter was much less efficient (about 30 fold lower) than from a small nuclear RNA promoter, U6 (17). In addition, the nuclear membrane presents a major barrier for DNA entry into the nucleus for transcription of the therapeutic gene. Since less than 1 % of the delivered plasmid DNA dose could gain entrance to the nucleus (18,19), efficient expression

system is needed to generate enough therapeutic RNA for efficient target down-regulation. Recently, endogenous small nuclear RNA, the U6 RNA, has been explored to drive the expression of short oligonucleotide, antisense (17), aptamers (20) and ribozyme (21). U6 RNAs are endogenous snRNAs, and they are involved in RNA splicing. The endogenous U6 RNA is very small (only 106 nt) and is constitutively expressed at a high level (0.5 million copies per cell). The U6 promoter is driven by RNA pol III and only a minimum of 24 nucleotides at the 5' end and 18 nucleotides at the 3' end are needed for expression of a stable transcript. The U6 expression system is particularly suitable for the expression of ribozymes (or antisense RNA) as the short flanking sequence would have minimal effect on the binding of the ribozyme to the target mRNA. However, reported attempts to employ the U6 system for the expression of any functional ribozymes intracellularly have been unsuccessful with unknown reason (20,21).

We showed that the U6 RNA promoter can be used to efficiently express functionally an active hammerhead ribozyme against c-neu. Transient transfection in ovarian cancer cells resulted in high level of ribozyme expression as well as specific inhibition of c-neu expression at both RNA and protein levels. In addition, non-viral delivery of the active ribozyme resulted in specific growth inhibition *in vitro* and *in vivo*.

MATERIALS AND METHODS

Plasmid Construction

Hammerhead ribozyme sequence against the human c-neu mRNA (position 2580-2604) was designed based on the fact that this region of c-neu mRNA is relatively accessible according to the RNA folding prediction (22). The active c-neu ribozyme (neuRz) and the mutant neu ribozyme (MRz) carrying the A to G mutation at position

15.1 (by ribozyme nomenclature (15)) are shown in Fig.1. For *in vivo* gene delivery, an additional control, pU6con (containing scramble flanking sequences) was included to eliminate any possible non-specific effect of DNA on tumor growth. This is because CpG motifs in plasmid DNA is known to stimulate immunological responses and may affect tumor growth *in vivo* (23-25). U6 expression plasmid pGEMmU6 was a kind gift from Dr. S. Noonberg (26). For cloning of pU6neuRz, pU6MRz and pU6con plasmids, PCR primers containing the cloning sites and part of the sequence of neuRz, MRz or con were synthesized. Using these primers, PCR-amplification was performed from another neuRz- or MRz-containing plasmid, which were also constructed by our laboratory. U6con was cloned from oligonucleotides with restriction sites at two ends. PCR-amplified fragments were cloned into pGEMmU6 between *Xho* I and *Nsi* I sites (Fig. 1). The sequence for cloning U6con was: 5'-ACCTAACGCTGACGATGAAGCAGGAGTGCCTGACTAAT CGCGAATAGCAGTGAGCT-3'. The sequences of the pU6neuRz, pU6MRz and pU6con clones were verified by automatic sequencing. For the combined plasmid, pU6neuRz-EGFP and pU6MRz-EGFP, a two-step cloning procedure was employed. Briefly, the pU6neuRz or pU6MRz plasmid was digested with *Bam*H I and *Eco*R I to obtain the insert that contains the U6 ribozyme and this insert was cloned into the promoterless open vector pEGFP-1 Promoter Reporter Vector (Clontech, CA) which contains a neomycin resistance gene and was previously digested with *Bgl* II and *Eco*R I. These resulting clones, which contain no promoter in front of the EGFP were subjected to the second round of cloning. CMV promoter from pEGFP-N' (Clontech, CA) was PCR amplified with primers carrying some CMV promoter sequences and the *Eco*R I and *Bam*H I cloning sites. This PCR fragment was then inserted into the clones from the first round of cloning to obtain the final combined plasmids, pU6neuRz-EGFP or pU6MRz-EGFP. The expression of the ribozymes and EGFP were checked by primer extension assay and observation of green fluorescence of cells after transfection, respectively.

Cell Culture and Transfection

Human ovarian carcinoma cell line, SKOV-3.ip1, was a kind gift from Dr. Mien-Chie Hung. Cells were maintained in DMEM with 10% fetal calf serum at 37 °C and 5 % CO_2. About 6.5 x 10^5 cells were plated on 6-well plates after trypsinization. After overnight seeding, a 5 h transfection in the absence of serum was performed with a liposome formulation optimized for this cell line. Cationic DC-Chol liposomes, DNA, JST-1 peptide (27) and protamine sulfate solution were mixed in the ratio of 3 nmol: 1 μg: 0.1 μg: 2.25 μg. DC-Chol liposomes were manufactured by this laboratory as described (28).

In vitro Transcription

Overlapping primers containing T7 RNA polymerase promoter for driving the *in vitro* transcription of the neuRz, MRz and c-neu target RNA were synthesized. Primer A : 5'-TAATACGACTCACTATAGGGACATGGTCTAACTGATGAG; Primer B : 5'-CTATGGCTGCCTTTCGTCCTCACGGACTCATCAGTT-3'; Primer C : same as Primer B except T is changed to C ; Primer D : 5'TAATACGACTCACTATAGGCCC TATGGCTGCCTCTTAGAC-3' and Primer E : 5'-GTTTTCCCGGACATGGTCTAAG AG-3'. Overlapping primers A and B or C were extended by PCR to generate the DNA template for *in vitro* transcription of neuRz or MRz, while primers D and E were for generating template for c-neu target RNA. The extensions were performed in PCR reaction buffer and PCR products were purified by chloroform : isoamylalcohol (49:1) extraction. In vitro transcription reactions were performed as described by Lowry *et al.* (29) and Zhang *et al.* (30). One microgram of DNA template, 1 mM NTP mix, 5 μM DTT, 10 units of human placental RNase inhibitor, 10 μCi of [α-^{32}P]UTP, 100 units of T7 RNA polymerase were mixed in 1x T3/T7 polymerase buffer. The mixture was incubated for 1 h at 37 °C, then 15 units of DNase I was added and incubated further for 20 min. The mixture was then purified by phenol-chloroform extraction and RNA was

precipitated at –80 °C in ethanol and then dissolved in DEPC-treated water and 50 % formamide.

In Vitro Cleavage Reaction

The cleavage reaction was adapted from Sarver *et al.* (31). Ribozyme and target RNA were added at ratios specified in the legend of Fig. 3. Cleavage reaction buffer (55 mM Tris-HCl, pH 7.5 and 1.5 mM Na-EDTA) was heated with the ribozymes and target RNA at 95 °C for 2 min followed by chilling on ice. The catalytic reaction was initiated by adding the required concentration of $MgCl_2$ and incubating at the desired temperature for different lengths of time. The reaction was stopped by adding equal volume of RNA loading buffer (95 % formamide, 1 mM EDTA, 0.1 % w/v xylene cyanol and bromophenol blue) and heated for 3 min at 65 °C. The cleaved products were separated by 7.5 M urea-PAGE. After electrophoresis, the gel was dried and quantitated using a phosphoimager. The sum of the intensities of the cleaved products was divided by the total sum of the intensities of uncleaved target and the cleaved products to calculate the percent cleavage.

Primer Extension

Cells were transfected with 6 μg of pU6neuRz or pU6MRz. Primer extension was performed as described previously (32). Briefly, at different days after transfection, total RNA was extracted from cells by Ultraspec™RNA isolation system (Biotecx Laboratories, Inc. TX). A U6-complementary oligonucleotide priming to the 3' end of the U6 RNA and the chimeric RNA was labeled with [γ^{32}-P]ATP and allowed to anneal with the total cellular RNA. Then primer extension was carried out using AMV reverse transcriptase at 45 °C. The reaction was quenched by adding EDTA. The synthesized cDNA was fractionated on a 12 % urea polyacrylamide gel and the result was recorded by X-ray autoradiography.

Northern Blot Hybridization

Total RNA was extracted six days after transfection. Eight micrograms of total RNA was resolved on a 0.8 % formaldehyde agarose gel and blotted onto a Nytran membrane (Schleicher and Schuell, NH). A 432 bp PCR fragment of the human c-neu cDNA (position 2181 to 2613) was labeled as the probe with [α-^{32}P]-dCTP by random priming (Boehringer Mannheim). Hybridization was performed at 42°C overnight in a solution containing 50 % formamide, 5 x SSC, 1 % SDS, 0.1 % Denhardt's, 0.25 mg/mg of yeast RNA and 50 mM sodium phosphate buffer (pH6.5). The blot was then washed twice with 5 x SSC-0.2 % SDS at 68 °C (about 20 min each). Autoradiography was then performed using an X-ray film.

Western Blot Analysis

Six days after transfection, cells were lysed with Lysis buffer containing 1 % Nonidet-P40, 150 mM NaCl, 1 mM EDTA, 10 mM sodium phosphate buffer (pH 7.2), 10 μg/ml aprotinin and 10 μg/ml leupeptin. Cell lysate was centrifuged at 14,000 rpm/min for 5 min at 4 °C. Supernatant was collected and the concentration of total protein was determined by Coomassie Plus Protein Assay Reagent (Pierce, IL) with BSA as a standard. Seven micrograms of total protein was loaded and resolved on a 3 % / 8 % SDS-polyacrylamide gel and then transferred to Protran membrane (Schleicher and Schuell, NH). The c-neu protein (p185) was detected by mouse monoclonal antibody against human c-neu; c-neu Ab-3 (Oncogene Science, Inc. NY) and peroxidase goat anti-mouse total Ig as the secondary antibody (Oncogene Science, Inc. NY). The blot was revealed using an Enhanced Chemiluminescence kit (Amersham, England). Percent of c-neu remaining was quantitated by densitometry.

Fluorescence-activated Cell Sorting and In vitro Growth Inhibition

SKOV-3.ip1 cells were transfected with the combined plasmid, pU6neuRz-EGFP or pU6MRz-EGFP. These combined plasmids ensured that all EGFP-expressing transfectants contained the ribozyme expression cassette. Two days after transfection, about 1.5 x 10^6 cells were trypsinized and washed 2 times with cold PBS containing 20 % fetal bovine serum and resuspended in about 1.5 ml of washing buffer. Cells were sorted by FACS (Flow cytometry service, UPCI, University of Pittsburgh). After sorting, cells were counted and seeded in triplicate with 30,000 cells/well in a 48 well plate. Two days after sorting, the cells were trypsinized, washed with DMEM medium containing 10 % FBS and the cell number was determined by counting. These procedures were repeated at different days and the percentage of growth inhibition was calculated with reference to the number of cells seeded (30,000/well).

Animal Model

Female CB17 SCID beige mice of 6 weeks old were used (Charles Rivers, Wilmington, MA). A subcutaneous solid tumor model were set up using SKOV-3.ip1. For inoculation, cells were trypsinized and then washed in Hanks buffer for three times. Mice were inoculated with about 3 x 10^5 cells by subcutaneous injection. The size of tumor was measured before the treatment each time.

Systemic Delivery of Naked DNA In Vivo

Plasmid DNA was diluted in 0.9 % NaCl (sterilized) and injected intravenously into the mice in a volume of 1.6 ml in about 8 seconds as described (33). Repeated injections of 10 μg of plasmid DNA were performed once every 2-3 days.

RESULTS

The Zucker's RNA folding method (34) was employed to design the hammerhead neuRz sequence targeting the human c-neu RNA in the tyrosine kinase domain which is known to be very important for its intracellular signal transduction. Fig.1 B shows the structure of the ribozyme (46 mer) and target c-neu RNA (35 mer) complex. Both flanking arms of the ribozyme contain 12 nts complementary to the c-neu RNA. This ribozyme was predicted to cleave at the 3' end of the CUC target site at position 2592 in the seventh exon of the c-neu, corresponding to the tyrosine kinase domain in p185 (35).

In vitro Cleavage of c-neu RNA by neuRz

A relatively short target RNA sequence (from 2579-2613) was chosen as a model substrate to test the cleavage activity of the designed ribozyme in vitro. Both the ribozymes and the target RNA were ^{32}P-labeled. A mutant ribozyme (MRz) was designed with an almost identical sequence as neuRz except the A to G mutation at position 15.1. This mutant ribozyme was expected to have no cleavage activity (36). The neuRz cleaved the c-neu target RNA efficiently, giving the cleavage products with the expected sizes of 21 and 14-mer under conditions of high temperature (60 °C) and high Mg^{2+} concentration (20 mM) (Fig. 2). More encouraging was the observation that no cleavage was found with the MRz under the same conditions (Fig. 2). The effect of ribozyme to target ratio on the cleavage reaction was studied (Fig. 3). The cleavage activity of the neuRz was efficient, resulting in about 70 % cleavage at ribozyme to target molar ratio of 0.1. This high cleavage activity was not increased even when the neuRz was present in two fold molar excess than the target RNA indicating that neuRz was performing at the maximal efficiency even at low ribozyme to target ratios. As a control, MRz demonstrated minimal background cleavage between 2-12 % in the range of ribozyme to

target ratios tested. It was further observed that short incubation time of only 20 minutes was required to achieve maximum cleavage and further incubation did not enhance cleavage significantly (data not shown). Even at physiological temperature (37 °C) and lower Mg^{2+} concentrations (2-6 mM), neuRz could result in 60-65 % cleavage activity at ribozyme to target ratio of 1:1 (data not shown).

Expression of U6neuRz and U6MRz in SKOV-3.ip1 Cells

The neuRz and MRz were cloned into the U6 expression vector (Fig. II-1). The resulting chimeric ribozyme RNAs carry the initial 25 nts of the human endogenous U6 gene (with A to C substitution at nt 24) and the *Xho* I site at the 5' end; with the *Nsi* I site and the terminator sequence at the 3' end (17,26). The expression of the chimeric U6neuRz and U6MRz in SKOV-3.ip1 cells was determined by primer extension, which could simultaneously detect the expression of the endogenous U6 gene also. As expected, primer extension for untransfected cells showed only the expression of the endogenous U6 RNA (106 nts), but no ribozyme expression. However, a very strong expression of the chimeric ribozyme RNA (100 nts) from the U6 expression cassette was detected two days after transfection of SKOV-3.ip1 cells with the pU6neuRz-or pU6MRz-liposome complexes. This high level of expression was only resulted from about 40 % transfection efficiency as determined by N'-EGFP (N'-enhanced green fluorescence protein plasmid) transfection and analyzed by flow cytometry (data not shown). This 40 % included cells that were both highly and weakly transfected. Endogenous U6 RNA level is known to be present at roughly 0.5×10^6 copies per cell (37). A conservative estimation of the average number of copies of chimeric ribozyme expressed at 48 hours after transfection was at least about 5×10^6 copies per cell (Fig. 4). These encouraging results demonstrate that the U6 expression cassette is a very efficient system for the high level expression of ribozymes in the cell as delivered by a non-viral vector.

Time Course of Ribozyme Expression

In order to investigate the duration of the chimeric RNA expression, the time course of chimeric U6neuRz expression was followed by primer extension at 1, 4 and 8 days after transfection into the SKOV-3.ip1 cells. As shown in Fig. 5, the expression of the chimeric RNA was still detectable up to 8 days after transfection, with an apparent half-life of less than 4 days. With the overwhelming expression on day 1, it was noticed that even at day 4, the amount of chimeric RNA in the transfected cells was still much higher than that of the endogenous U6 RNA.

Down-regulation of c-neu RNA in SKOV-3.ip1 cells

SKOV-3.ip1 is a highly metastatic subline of SKOV-3. Both the parental and subline overexpress c-neu RNA. Northern blotting (Fig. 6) for c-neu RNA showed two major bands as reported previously (38). A 7.5 kb major transcript was detected in addition to the 4.8 kb transcript. This very same pattern had been observed using labeled probes of various length (data not shown). When compared to pU6MRz transfectant, c-neu RNA was reduced to about 50 % at day 6 after pU6neuRz transfection, which demonstrated that the chimeric U6neuRz was catalytically active and specifically down-regulated the target c-neu RNA in SKOV-3.ip1 cells. More interestingly, the large 7.5 kb transcript which may be resulted from the alterations associated with gene amplification observed in some ovarian cancer cell lines (38) was also down-regulated by U6neuRz. The chimeric U6MRz also seemed to partially down-regulate c-neu RNA (compared to lane C in Fig. 3) but not as much as U6neuRz, which is believed to be resulted from the antisense effect of the U6MRz. Smearing of RNA on the northern blot could be resulted from the degraded c-neu mRNA in transfected cells.

Dose-dependent Down-regulation of c-neu Protein (p185)

The c-neu protein was detected by Western blot analysis as a single band at 185 kD. Cells transfected with an increasing dose of plasmid pU6neuRz showed a reduced amount of p185 protein expression (Fig. 7). With 8 or 16 μg of plasmid pU6neuRz, about 39 - 42 % (n=5) of p185 was down-regulated. With 32 μg DNA dose, U6neuRz specifically down-regulated p185 by 55 % (n=3). However, U6MRz only showed minimal p185 down-regulation of about 10-20 % in the DNA doses tested which qualitatively agrees with the partial down-regulation of c-neu RNA by U6MRz in Northern blot result. With less than 8 μg of plasmid, no down-regulation of p185 at day 6 has been observed (data not shown).

In vitro Growth Inhibition by U6neuRz

For unsorted populations after transient transfection, growth inhibition could not be easily detected by both anchorage-dependent and anchorage-independent growth (data not shown). Since transient transfection was purposely employed to avoid any potential artifacts from clonal variations of the stable clones, the inability to detect any significant growth inhibition was probably due to the low transfection efficiency of SKOV-3.ip1 cells. Moreover, with transient transfection, we would only expect transient phenotypic changes if there was any. Taking these factors into consideration, transient transfectants (by the combined plasmids, pU6neuRz-EGFP and pU6MRz-EGFP) were sorted by FACS at day 2 after transfection. The highly green fluorescent cells were collected, replated and counted every 2 days (Fig. 8). At day 2 after sorting, a significant difference in growth rate (average of 20 % growth compared to 60 % growth) was observed between the pU6neuRz-EGFP and the pU6MRz-EGFP groups. As expected, after additional 2 days, the difference became smaller, being 39 % and 57 %, respectively. With additional 5 days (total of 9 days after sorting), the growth rate of the transfectants seemed to resume but still there was a significant difference between them. It was observed that both the

pU6neuRz-EGFP transfectant and the pU6MRz-EGFP transfectant grew much faster compared to the earlier time point after sorting (8.8- and 11-fold, respectively). This is likely to be due to the loss of plasmid DNA from the transfectants or reduced expression of the ribozymes (Fig. 5), while the effect of down-regulating c-neu still results in preferential growth retardation in the pU6neuRz-EGFP transfectant. Although the phenotypic effect shows a transient trend, the active chimeric ribozyme did exert a significant and specific growth inhibition of SKOV-3.ip1 cells.

In vivo Growth Inhibition

Using the hydrodynamics-based gene delivery method, mice were injected with pU6nueRz or pU6MRz plasmid DNA once every 3-4 days. Treatment was started on day 3 after tumor inoculation. The tumor size was monitored every time before injection. At day 19, experiment was terminated due to the ulceration of tumors in some mice. A total of 6 injections was given to each treatment group. The distribution of tumor size at the end of the experiment was shown (Fig. 9). The mean size of tumors in the pU6neuRz group (24.08 ± 3.16 mm^2, $p = 0.059$) was significantly smaller than that of the pU6MRz control (33.41 ± 2.48 mm^2, $p = 0.857$), the pU6con control (32.73 ± 2.69 mm^2, $p = 0.502$) and the untreated group (30.66 ± 1.09 mm^2). The p values (Student t-test) for statistical analysis are < 0.1. This partial inhibition of tumor growth suggested that the chimeric U6neuRz could be potentially used as a therapeutic agent for the treatment of ovarian cancer.

DISCUSSION

Down-regulation of c-neu expression in cancer cells is very challenging for 2 main reasons. First, c-neu mRNA has a very long half-life. The turnover of c-neu mRNA

is very slow and exhibits a biphasic kinetics (39). It has a long half-life of about 7 hours in the first phase; and the remaining 40 % of mRNA then stayed undegraded even after transcription arrest lasted for 24 h (39). Second, very high level of over-expression is observed in cancer cells due to gene amplification. Up till now, only stable clones or adenoviral vector expressing antisense or ribozyme against c-neu are efficient enough to result in successful down-regulation of c-neu expression. Therefore, in order to use non-viral vector (which has limited transfection efficiency) to abrogate c-neu expression, a very efficient expression system for therapeutic RNA, such as antisense or ribozyme, is needed. In this study, we explored the feasibility of developing a functionally active hammerhead ribozyme against c-neu using a highly efficient non-viral vector, the U6 expression system. Extremely high level of ribozyme expression (5 x 10^6 copies/cell) was observed by transient transfection, which was accompanied by specific down-regulation of c-neu expression (both at RNA and protein levels). Non-viral gene transfer resulted in significant growth inhibition of ovarian cancer cells *in vitro* and *in vivo*.

Our results demonstrated that this potent non-viral expression system is suitable for the expression of a functionally active hammerhead ribozyme. We have shown that U6 promoter is much more efficient and specialized than a strong viral promoter (CMV) which is most commonly used (17). The U6 expression system has also been explored to express antisense RNA (17), aptamer and ribozyme (20,21). We previously demonstrated that a chimeric U6-E6/E7 could result in specific inhibition of tumor growth in an animal model, indicating the potential clinical value of this U6 expression system (17). Although functional antisense and aptamer sequences could be expressed from the U6 promoter *in vivo*, ribozymes expressed in the context of U6 RNA has been reported with no functional activity in intact cells or only a minimal target down-regulation (20,21). The reason for this is unclear, though nuclear confinement of ribozymes (thus limiting the interaction of ribozyme with the target RNA in the cytosol) was suggested as one of the possibilities (20). However, co-localization of ribozyme with the target RNA at the splice site may

actually be an advantage for ribozyme-target interaction. Besides being able to express a large amount of stable RNA, the U6 promoter should be suitable for expressing short ribozymes, as endogenous U6 RNA itself is quite small. Moreover, there is a theoretical limit to the length of an antisense to be produced from the U6 system because the premature termination signals (TTGTT and TTATT) would appear once every 256 nucleotides. Therefore, this U6 system may be more suitable to express short ribozymes rather than long antisense sequences. Unlike other RNA polymerase III-dependent promoters such as tRNA promoter, U6 promoter does not contain any internal control region. Therefore, short ribozyme with minimal flanking sequences at two ends, can be expressed with minimal interference to the ribozyme activity.

For the design of any specific antisense or ribozyme, target RNA accessibility is one of the most important issues. Although it is difficult to closely simulate the intracellular environment, different computer programs have been established for the prediction of RNA folding with acceptable accuracy (22). The secondary structure of the human c-neu RNA was predicted by Zuker's model (40). The cleavage site and the target sequence chosen are predicted to locate right on the bulge and a short loop, which implies that this sequence may be rather accessible (RNA folding structure not shown) for base-pairing with the ribozyme. In agreement with these predictions, the *in vitro* cleavage of target c-neu RNA by neuRz was efficient (up to approximately 70 %), even under conditions mimicking the physiological temperature and Mg^{2+} concentration. With target RNA 10 fold in excess, the catalytically active neuRz could still exhibit close to maximal cleavage activity (Fig. 3). Reason for the neuRz being unable to cleave the residual 30 % of the target RNA is unknown. Addition of fresh neuRz at the end of incubation did not result in more target cleavage (data not shown). One possibility is that 30 % of the target RNA could be folded into a conformation that was inaccessible to the ribozyme. Mutation of A to G at position 15.1 rendered the ribozyme catalytically inactive (Fig. 2 & Fig. 3), which is in agreement with previous reports (41-43).

SKOV-3.ip1 is a subline of SKOV-3 ovarian carcinoma cells. It has a higher degree of malignancy and a higher level of p185 expression than the parental cell line (44). Transient transfection resulted in extremely high level of ribozyme expression (at least 5 x 10^6 copies/cell) (Fig. 4). This agrees with the reported efficiency of this U6 expression system (20,26). In fact, the expression efficiency was higher than the endogenous U6RNA. Furthermore, the expressed ribozyme was rather stable, having a relatively slow decay with an estimated half-life of about 4 days. Even at 4 days after transfection, the amount of U6neuRz in the cells approximately equaled to that of the endogenous U6 RNA (Fig. 5). The chimeric ribozymes were expected to be rather stable as the U6 expression cassette contains sequences for a 5' hairpin and 5' γ-monomethyl phosphate capping, which confers extra stability to the expressed sequence (20,26). By dot blot analysis, it was estimated that about 8.9 x 10^3 - 1.8 x 10^4 copies of c-neu mRNA per cell was expressed in SKOV-3.ip1 cell (data not shown). Therefore, the ribozyme was expressed in 500-fold excess of the target RNA in the cells.

Specific inhibition of c-neu expression and growth rate by U6neuRz, but only minimally by U6MRz, were observed (Fig. 6 to 8) when cells were transiently transfected with a ribozyme-expressing plasmid. These indicated that the observed activity of U6neuRz must be largely due to its cleavage activity.

There are several possible reasons for the observed partial down-regulation of c-neu expression as well as partial growth inhibition *in vitro* and *in vivo*: 1) relatively low *in vitro* transfection efficiency of this cell line (about 40 %) even with an optimized lipofection formulation. The partial growth inhibition *in vivo* suggested that the *in vivo* transfection efficiency might still be suboptimal. This was in agreement with our observation that no growth inhibition was observed for unsorted cells *in vitro* (data not shown). Therefore, further improvement of the gene delivery method may allow the achievement of an improved therapeutic effect. 2) in order to achieve significant growth inhibition effect on c-neu over-expressing tumors, prolonged expression of antisense or

ribozyme may be required as inhibition of c-neu seems to result in a cytostatic rather than a cytotoxic effect(45,46). It is possible that cell growth will resume once the ribozyme expression subsides. This notion is supported by the fact that tumor growth inhibition was observed only after repeated injections of an adenoviral vector expressing a c-neu ribozyme (47). Besides this adenovirus delivery study, all other *in vivo* studies employing antisense RNA or ribozyme for c-neu down-regulation have used stable clones. 3) long half-life of c-neu mRNA makes complete down-regulation difficult. 4) the chimeric U6neuRz cannot cleave c-neu mRNA in intact cells as efficient as it does *in vitro*. The target mRNA could be folded differently and bound by proteins in the cell, rendering the target site less accessible to the ribozyme than in the isolated *in vitro* condition. The hammerhead ribozyme expressed in the context of flanking U6 sequences may also be folded differently than the free ribozyme. Its cleavage activity may be further compromised by the complex intracellular environment, which is very different from a simple buffered solution used *in vitro*. Previously, hammerhead ribozymes with a high catalytic activity *in vitro* and in cell extract, but not in intact cells, have been reported (48,49).

The role of c-neu in tumorigenesis is still not totally understood. Previously, antisense approach seemed to be able to answer the question. However, it is now realized that growth inhibition resulted from antisense treatment could be contributed by the non-specific effect of oligonucleotides. Similar non-specific effects could occur to selection of stable clones expressing antisense and ribozymes. However, with the advantage offered by the ribozyme and the respective disabled mutant control (which has only a single base mutation at the catalytic core), we are able to demonstrate conclusive phenotypic effects of transient down-regulation of c-neu. The fact that sorted pU6neuRz-EGFP transient transfectants showed significant growth inhibition in comparison with with the pU6MRz-EGFP transfectants clearly demonstrated that U6neuRz was active and target-specific

within intact cells. Moreover, specific *in vivo* growth inhibition of tumor by U6neuRz further confirmed the role of human c-neu in cancer cell growth.

In conclusion, we have demonstrated that the U6 expression system is very efficient for the expression of a functionally active hammerhead ribozyme, which can down-regulate a target with a long half-life. Non-viral delivery of U6neuRz results in growth inhibition of ovarian cancer cells *in vitro* and *in vivo*. Thus, U6 expression system may provide an efficient alternative gene therapy approach for down-regulating specific genes *in vivo*. We are exploring its use in other gene therapy applications.

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Legends to the Figures

Fig.1A. Schematic diagram of the ribozyme expression cassette in the U6 expression vector. Using oligonucleotides, genes for the expression of c-neu ribozyme (neuRz) or mutant c-neu ribozyme (MRz) were cloned between the *Xho*I and *Nsi* I sites. The U6 cassette was inserted between *Bam*H I and *Eco*R I sites of pGEM-1 plasmid. B. A schematic representation of ribozyme and c-neu RNA complex. Structure of the ribozyme is shown with the conserved catalytic core flanking complementary sequence to c-neu RNA (base pairs 2580-2604). The cleavage site is at the 3'end of the CUC trinucleotide. MRz differs from neuRz by A to G mutation at the indicated position.

Fig.2. *In vitro* cleavage reaction. Autoradiogram showing the cleavage of the c-neu transcript with the in vitro transcribed neuRz (lane 1), MRz (lane 2), neuRz only (lane 3), and target only (lane 4). The neuRz and c-neu transcript has a length of 46 and 35 nt respectively; and the cleavage products P1 and P2 are 21 and 14 nt long respectively. The cleavage reaction was performed at 60°C in 20 mM $MgCl_2$ for 1 h.

Fig.3. *In vitro* cleavage rates of c-neu RNA with different ribozyme/target RNA molar ratios. Reaction mixture containing increasing number of moles of neuRz (■) and MRz (●) with constant target concentration (200 pmoles) was incubated at 60°C with 20 mM $MgCl_2$ for 1 h.

Fig.4. Detection of the U6neuRz and U6MRz expression by primer extension. Two days after transfection, total RNA was extracted from pU6neuRz (A) and pU6MRz (B) transfected SKOV-3.ip1 cells, and untransfected cells was used as a control (C). Arrows

indicate the size of the endogenous U6 RNA (106 nt), the U6neuRz and U6MRz (100 nt). The experiment was repeated three times with similar result each time.

Fig. 5. Time course of U6neuRz expression by primer extension. Total RNA was extracted from SKOV-3.ip1 cells 1, 4 and 8 days after transfection with 6 μg of pU6neuRz. Expression levels of U6neuRz at day 1, 4, and 8 are shown in lane A, B, and C respectively. Untransfected control (lane D) showing only the expression of endogenous U6 RNA (106 nt) but not the U6neuRz (100 nt). Lane C and D are shown in longer exposure for more clarity.

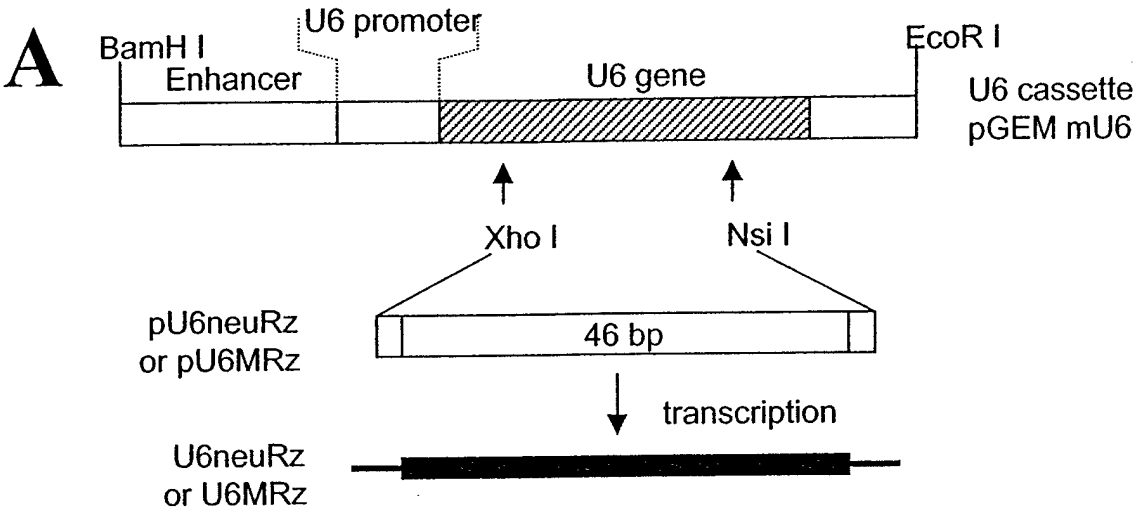
Fig.6. Northern blot showing the down-regulation of c-neu mRNA in SKOV-3.ip1 cells by U6neuRz. Plasmid pU6neuRz (A) and pU6MRz (B) were delivered into the cells by lipofection. Total RNA from untransfected cells was used as a control (C). Total RNA was extracted six days after transfection. A 432 bp human c-neu gene fragment was labeled with [α-^{32}P] dCTP and used as the probe. RNA loading is shown in the lower panel. Experiment was repeated three times with similar results obtained each time.

Fig.7. Down-regulation of c-neu protein (p185) by pU6neuRz transient transfection. A) Western blotting showing c-neu down-regulation in SKOV-3.ip1 cells. Seven μg of total protein was loaded in each lane. The c-neu protein level after transfection with pU6neuRz and pU6MRz and untransfected control are shown. Arrow indicates the size of c-neu (185 kD). B) Graphical representation showing the dose-dependent down-regulation of p185. The percentage of c-neu protein remaining six days after transfection in one experiment. Similar results have been observed in several experiments.

Fig. 8. FACS of cells transfected with the combined plasmids and preferential growth inhibition of pU6neuRz-EGFP transfectant compared to pU6MRz-EGFP transfectant. Experiment was repeated with triplicate or more with similar results obtained.

Fig. 9. *In vivo* delivery of plasmid DNA carrying the ribozyme constructs. Multiple injections (once every 2-3 days) were performed. The size of s.c. SKOV-3.ip1 tumors were measured each time before injection. U6con was a scramble control. Untreated control (n = 7), pU6con (n = 6), pU6neuRz (n = 8) and pU6MRz (n = 8). The p values for each group in comparison to the untreated group (*) are shown in the bracket.

Fig. 1


A
BamH I
Enhancer
U6 promoter
U6 gene
EcoR I
U6 cassette
pGEM mU6
Xho I
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pU6neuRz
or pU6MRz
46 bp
transcription
U6neuRz
or U6MRz

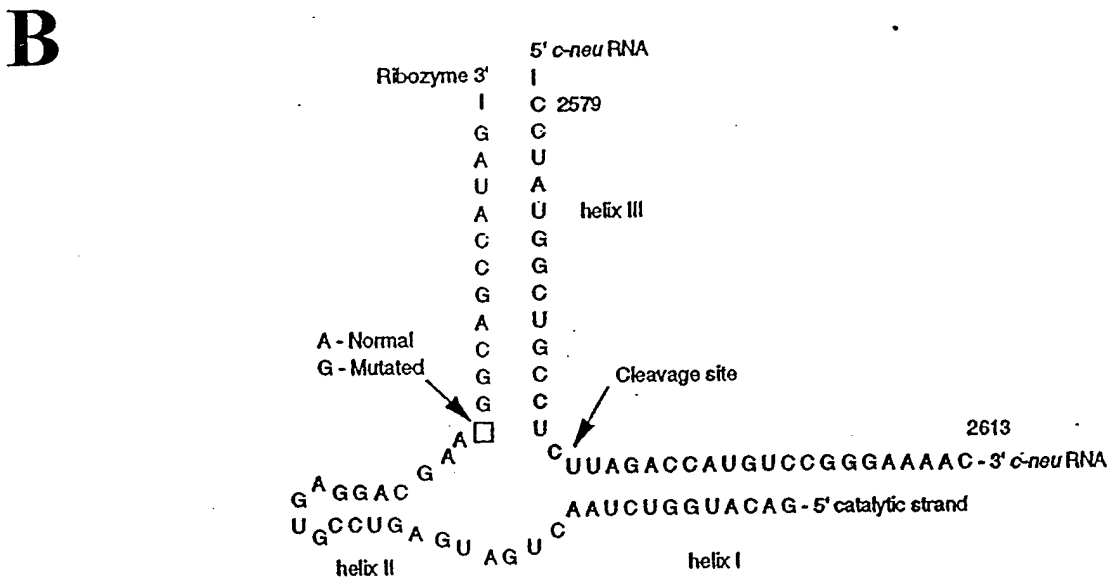

B
5' c-neu RNA
Ribozyme 3'
C 2579
helix III
A - Normal
G - Mutated
Cleavage site
2613
UUAGACCAUGUCCGGGAAAAC - 3' c-neu RNA
AAUCUGGUACAG - 5' catalytic strand
helix II
helix I

Fig. 2

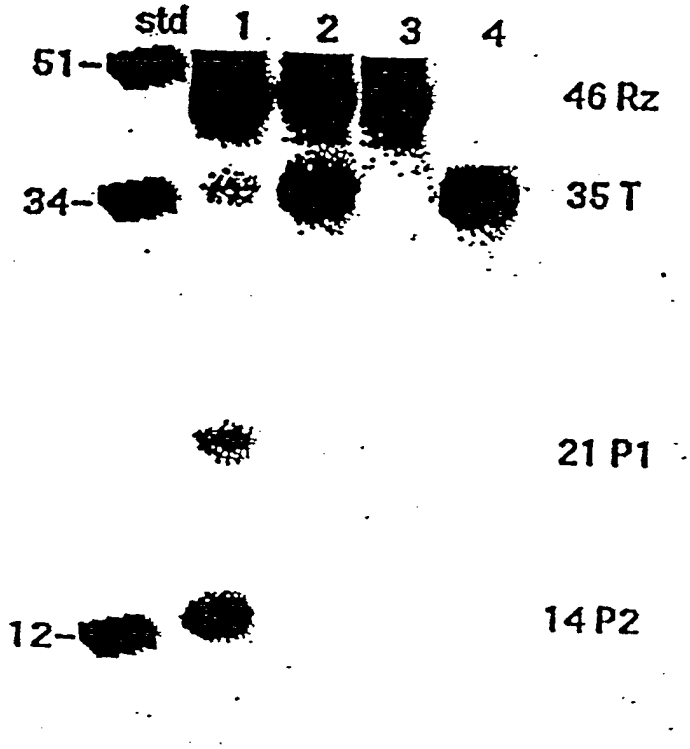


Fig. 3

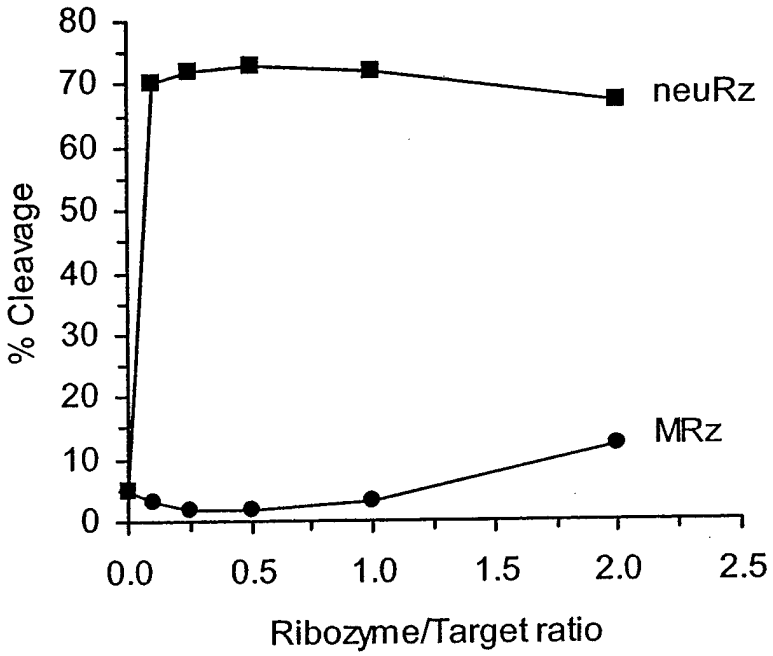

80
70
60
50
40
30
20
10
0
% Cleavage
neuRz
MRz
0.0
0.5
1.0
1.5
2.0
2.5
Ribozyme/Target ratio

Fig. 4

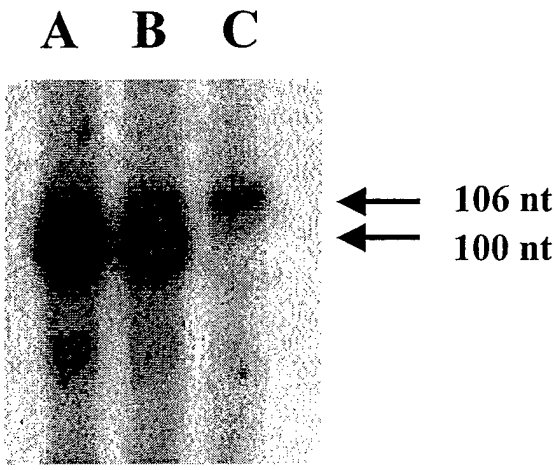
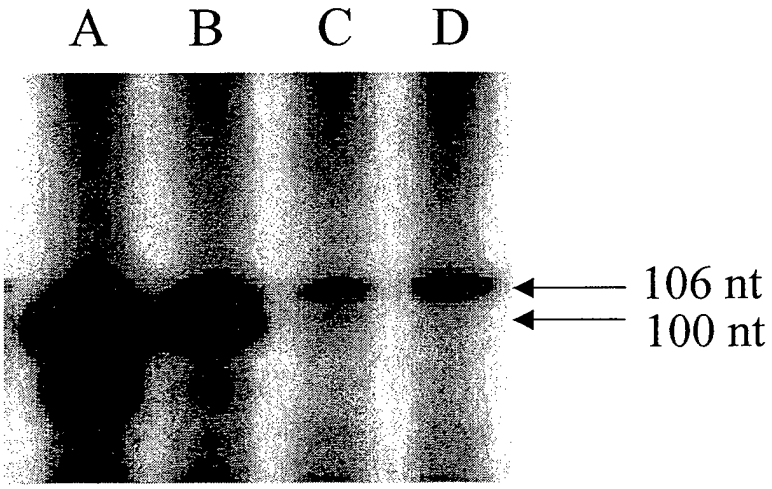


Fig. 5



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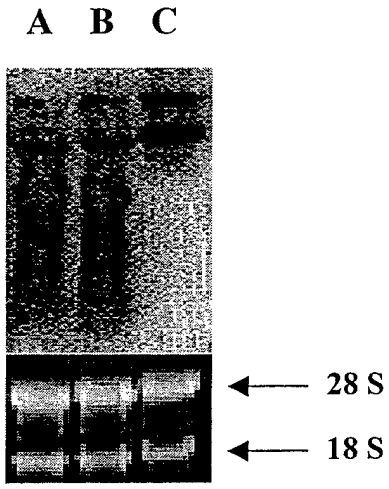


Fig. 7

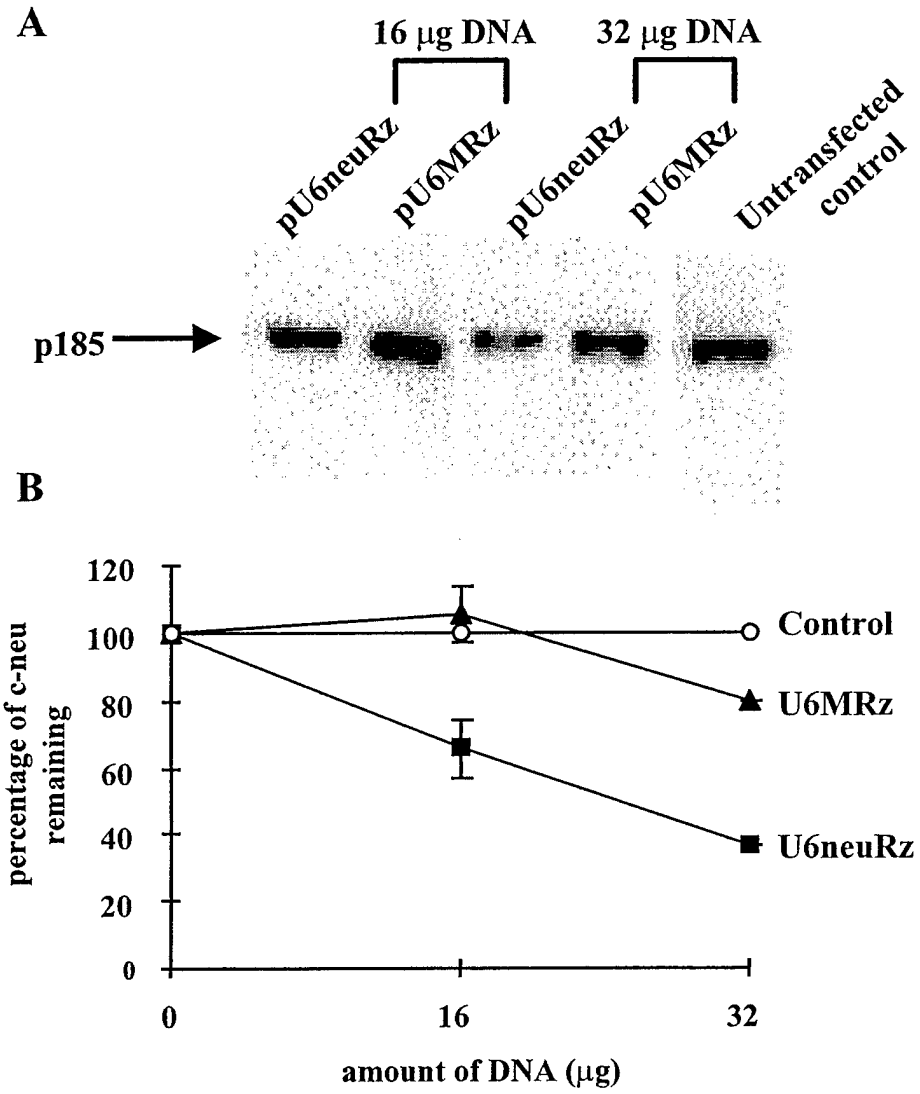

A
16 μg DNA
32 μg DNA
pU6neuRz
pU6MRz
pU6neuRz
pU6MRz
Untransfected control
p185
B
120
100
80
60
40
20
0
percentage of c-neu remaining
Control
U6MRz
U6neuRz
0
16
32
amount of DNA (μg)

Fig. 8

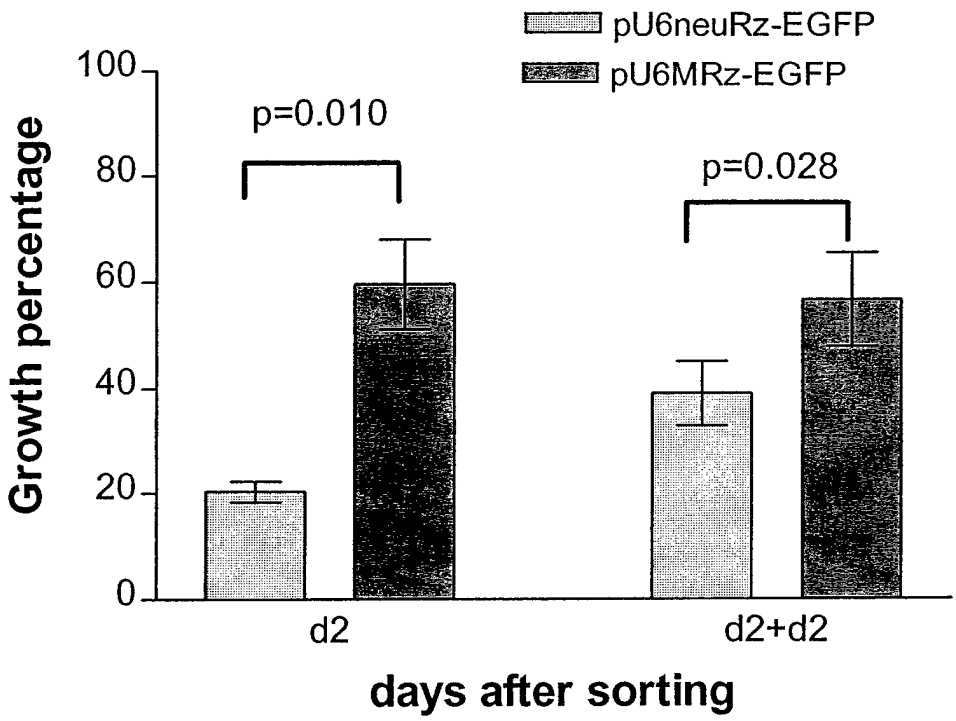
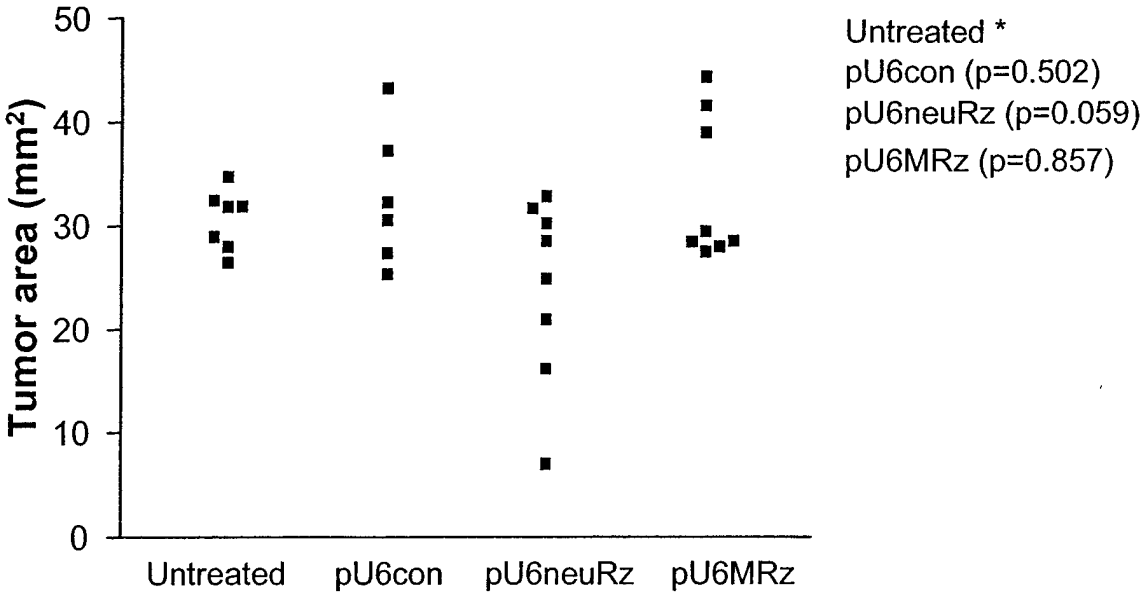

pU6neuRz-EGFP
pU6MRz-EGFP
p=0.010
p=0.028
100
80
60
40
20
0
Growth percentage
d2
d2+d2
days after sorting

Fig. 9


50
40
30
20
10
0
Tumor area (mm²)
Untreated
pU6con
pU6neuRz
pU6MRz
Untreated *
pU6con (p=0.502)
pU6neuRz (p=0.059)
pU6MRz (p=0.857)

Running head: PSYCHOSOCIAL INFLUENCES ON CANCER PROGRESSION

Psychosocial Influences on Cancer Progression: Alternative Cellular and Molecular Mechanisms

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Abstract

Considerable interest in the biobehavioral pathways linking stress and cancer as well as the identification of modifiable risk factors has increased research examining psychological adjustment, biological responses, and cancer outcomes. Although most of this work has focused on how stress affects processes such as immune surveillance that govern survival of tumors, less attention has been directed at how stress contributes to somatic mutation and genomic instability. Progress in this area may be facilitated by considering how stress affects events that modulate development and accumulation of somatic mutations in addition to those affecting survival of tumor cells. It is possible that a sharper focus on other relevant biological processes such as increases in DNA damage, alterations in DNA repair, and inhibition of apoptosis, may explain more of the variance in disease outcomes.

Key Words: STRESS, CANCER, DNA DAMAGE, DNA REPAIR, APOPTOSIS, SOMATIC MUTATION

Cancer is the second leading cause of death in the United States [1]. More than 1.2 million people were diagnosed with cancer in 1998 and more than 500,000 people died of it, based on National Cancer Institute (NCI) Surveillance, Epidemiology, and End Results (SEER) data [2]. Men living in the United States have a one in two lifetime risk of developing cancer and women's lifetime risk is one in three [2]. Similarly, the estimated number of new cancer cases in member states of the European Union was more than 1.3 million and the number of deaths was about 900,000 [3]. Although cancer death rates in the US have declined nearly three percent between 1991 and 1995 [2], cancer remains a serious and stressful disease.

Considerable interest in the biobehavioral pathways linking stress and cancer as well as the identification of modifiable risk factors has increased research examining psychological adjustment, biological responses, and cancer outcomes. However, research examining the affects of psychosocial variables such as stress or depression on the etiology or progression of cancer continues to report mixed effects [4-9]. Efforts to identify specific mechanisms underlying or affecting cancer course have met with limited success. Most of this work has focused on how stress affects processes such as immune surveillance that govern survival of tumors. Less attention has been directed at how stress contributes to somatic mutation and genomic instability. Progress in this area may be facilitated by considering how stress affects events that modulate development and accumulation of somatic mutations in addition to those affecting survival of tumor cells. It is possible that a sharper focus on other relevant biological processes such as increases in DNA damage, alterations in DNA repair, and inhibition of apoptosis, may explain more of the variance in disease outcomes. This review considers a

growing body of literature that bears on presumed mediators of psychosocial modulation of cancer course and research on cellular and subcellular aspects of stress.

To understand how psychosocial factors influence the etiology or progression of cancer, researchers need to examine psychosocial influences on basic mechanisms of mutagenesis and carcinogenesis. While we acknowledge the strong impact of various health-related behaviors on cancer (e.g. smoking, diet, sedentary lifestyle), review of health behaviors and their contribution to cancer are beyond the scope of this review. We begin with immune surveillance because it has been the primary model of stress and cancer modulation over the past 10-20 years.

IMMUNE SURVEILLANCE

The concept of immune surveillance against neoplastic growth dates back to early part of the twentieth century and is based on the idea that various components of the immune system are responsible for host defense against the emergence and growth of tumor cells [10]. The theory generally suggested that the immune system was capable of recognizing tumor cells, and initiating a specific cytotoxic response against them. Host resistance against neoplasia was thought to be T-cell dependent and since tumor cells reliably expressed recognizable tumor associated antigens (TAA), immune defenses against tumor cells should resemble responses to viral infection. The implication of this theory was that immunosuppression was associated with, and should precede, the development of tumors [10].

Although there is some evidence that is consistent with the immune surveillance hypothesis, particularly in animal models, most research in humans suggests that the hypothesis has major limitations. For example, it is not clear that all tumors express TAA or at what point in the transformation process these antigens emerge. Additionally, tumors may shed these TAA or actively suppress numerous immune cells [11]. Finally, systemic immunosuppression is not

reliably related to tumor development and malignancy, especially the more common cancers of the breast, colon, and lung [7].

Because of these and other difficulties, the immune surveillance hypothesis has undergone significant revision. The focus on T-cells as primary effector cells has been de-emphasized in favor of a stronger role for natural killer (NK) cells because of their ability to non-specifically recognize and lyse tumor cells *in vitro*, especially blood-borne tumors [12]. This, together with increasingly clear evidence that stress affects NK trafficking and cytotoxic capacity independent of cancer [13], has led to the development of biobehavioral models of cancer [e.g. 14-16]. These models suggest that psychosocial factors such as chronic stress, depression, or lack of social support contribute to cancer initiation or progression by suppressing NK cell numbers or lytic function. These theories further suggest that much of the variance in these relationships is modifiable and that psychosocial interventions aimed at providing education and support should reduce distress and corresponding suppression of NK activity. However, as will be discussed, evidence of restorative effects of psychosocial interventions is mixed [17].

While there is convincing evidence for the role of NK cells in host resistance to tumors in animals, fewer studies show a clear role for NK in host resistance against tumors in humans [18]. Although NK cells can lyse allogenic tumor cell lines without prior sensitization, they are unable to lyse freshly isolated autologous tumor cells unless cultured with IL-2 [18]. Additionally, although there is evidence that NK cytotoxic activity (NKCA) is reduced in certain tumors [18], it is not known if this is the cause or the result of tumor burden. Tumors may themselves produce local suppression factors that may disrupt NK function [11].

Several correlational studies have examined the effects of psychosocial variables on NK number and function in cancer patients and data are consistent with the NK surveillance

hypothesis. For example, Levy et al., [19] found that 51% of the variance in NKCA in breast cancer patients was accounted for by psychological adjustment, lack of social support, and fatigue. Additionally, breast cancer patients' perceptions of social support and how actively they sought social support significantly predicted NKCA [20]. Baseline NKCA predicted greater spread to axillary lymph nodes and better NKCA at follow-up predicted disease-free survival [21]. Andersen et al., [22] found that intrusive and avoidant thoughts about cancer negatively predicted NKCA and that intrusive thoughts negatively predicted NK response to recombinant interferon gamma (rINF-γ). Surprisingly, the majority of subjects did not show an NK response to recombinant interleukin-2 (rIL-2). Tajima et al., [23] report a significantly lower absolute number of NK in patients compared to controls, that NKCA was reduced in patients compared to controls, and that untreated or relapsed patients had lower NKCA than patients off treatment. These studies provide moderate but consistent evidence that psychological factors such as stress influence NK activity in cancer patients and that these alterations in NK activity are related to important disease processes.

The most compelling data concerning the role of immune surveillance in cancer patients comes from a randomized clinical trial of a six week structured psychoeducational intervention for malignant melanoma patients [24]. Following the intervention, patients exhibited significant improvement in NKCA at six-month follow-up and higher NKCA at baseline was related to lower recurrence rates [25]. Unfortunately, these findings are not representative of the intervention literature as a whole. A recent meta-analysis reviewed evidence for psychological modulation of immune responses in humans from studies of psychosocial interventions and concluded that interventions have a relatively modest effect on immunity [17]. Moreover, the meta-analysis revealed no evidence to support the assertion that interventions can stimulate

beneficial immune changes in medical populations. The authors suggest that powerful biological forces (e.g. treatment protocols) may overwhelm immune changes induced by the intervention [17]. Additionally, it is not clear whether these changes in immune system activity are of sufficient magnitude for cancer to develop or progress [26].

DNA DAMAGE

The immune system and its supportive function can be thought of as affecting the likelihood that somatic mutations survive, accumulate, and eventually induce tumor growth. However, another important level of analysis is subcellular and relates to whether stress affects the likelihood of DNA damage, somatic mutation, and genomic instability. Damage to DNA from oxygen free radicals (OFR) is perhaps the most common form of DNA damage [27] and is not only important in tumor initiation but is also prominent in all phases of tumor promotion and disease progression [28, 29]. However, current biobehavioral models of cancer stress fail to account for oxidative damage as a plausible mechanism linking psychosocial variables and cancer. OFR result from exposure to pollution or ionizing radiation and are generated as by-products of inflammation and normal oxygen metabolism [29]. OFR damage DNA by attacking either its nucleotide residues or sugar backbone. OFR also damage other cellular components, such as lipids and proteins, that produce reactive intermediaries, which in turn, form mutagenic adducts with DNA [30].

Research suggests that oxidative damage to DNA (i.e. alteration of the coding sequence or functional properties of DNA) plays a role in mutagenesis and increases risk for developing cancer [29, 28, 31]. Research has demonstrated that unrepaired or misrepaired oxidative lesions are mutagenic and cause miscoding [32], single strand breaks, and induction of microsatellite instability [33]. The mutations induced by many of these lesions are commonly observed in

mutated oncogenes & tumor suppressor genes [34]. Oxidative lesions may also cause deletions, double strand breaks, chromosomal aberrations, micronuclei formation, damage to histones, aberrant signal transduction, and altered gene expression [35]. Additionally, oxidative lesions are mitogenic, which increases the probability of mutation through an increased number of replication cycles [31]. These observations suggest that increasing oxidative damage figures prominently in the etiology of many cancers [34].

The reported five- to seven-fold interindividual differences in excreted oxidative damage products suggest that there may be modifiable sources of OFR [29]. Stress has been shown to damage DNA at the molecular [36] and chromosomal levels in rats [37, 38] and Irie, Asami, Nagata, Miyata, & Kasai [39] recently demonstrated that renal levels of 8-OH-dG, a common biomarker of oxidatively damaged DNA could be classically conditioned using a conditioned taste aversion paradigm. In one of the few studies examining psychological variables and oxidative damage in humans, Irie, Asami, Nagata, Miyata, & Kasai [40] found that levels of 8-OH-dG were negatively related to the Tension-Anxiety and Depression-Dejection scales of the Profile of Mood States (POMS) in males, but positively related to Depression-Dejection scores in women. Further, 8-OH-dG levels were increased in subjects who experienced the loss of a close family member within the previous three years when compared to those without a similar loss experience. These data suggest that oxidative damage may be related to mood and suggest that there may be sex differences in the generation of oxidative lesions. Finally, Forlenza, Latimer, & Baum, [41] found increased levels of DNA repair prior to exogenous damage suggesting that stress increased endogenous damage to DNA.

It is possible to directly measure oxidative lesions with a variety of assay systems in both urine and blood [e.g. 42, 43]. Blood measures of oxidative damage provide information on

steady state levels of oxidative adducts to DNA and urine measures reflect the rate of damage [29]. Common assay techniques include High Performance Liquid Chromatography (HPLC) with electrochemical detection and enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies to oxidative lesions such as 8-hyrdoxyguanine. Measurement of biomarkers of oxidative damage can be a valuable tool for behavioral medicine and other biobehavioral studies of the impact of stress, adjustment, and other psychological variables in cancer onset as well as the impact of psychosocial interventions on disease progression.

DNA REPAIR

If damage to DNA is not repaired prior to replication, resulting errors can become fixed in the genome and give rise to somatic mutations. Fortunately, mammalian cells have evolved mechanisms to recognize and repair many forms of damage to DNA. There are multiple repair pathways each specializing in the repair of a specific type of damage. For example, mismatch repair acts as a post-replication "spell-checker" and fixes errors made by DNA polymerase during replication. Base excision repair focuses on single damaged bases and nucleotide excision repair (NER) targets a large variety of bulky lesions including pyrimidine dimers, DNA cross-links, and 6-4 photoproducts [44].

Nucleotide excision repair (NER) is a multi-step process that removes a broad array of DNA lesions including UV and chemical damage [45]. Proteins from at least 30 genes are necessary to complete the repair process, eleven of which have been cloned. Although all of the details have not yet been worked out, there are five basic steps involved in the NER pathway: recognition of DNA alteration or damage, incision of 27-29 bp of DNA around the lesion, excision of the damaged portion, re-polymerization of the correct sequence, and finally, ligation of the DNA strands [29].

Previous studies have shown that there is a substantial amount of variability in DNA repair capacity [46]. Wei, Matanoski, Farmer, Hedayati, & Grossman [47] reported that the distribution of DNA repair capacity in the peripheral T-lymphocytes of their subjects was approximately normal with a five-fold variation among individuals. Given this large amount of variability, it is reasonable to examine psychosocial variables that might explain differences in rates of repair.

There is also evidence that DNA repair is associated with cancer. People with recessively-inherited DNA deficits are cancer-prone and studies suggest that unrepaired DNA damage has high carcinogenic potential [48]. For example, individuals born with xeroderma pigmentosum (XP) are extremely prone to cancerous skin lesions (carcinoma and melanoma) because they are unable to repair damage caused by exposure to UV light. Studies show that XP patients show a mutation in the genes coding for overall genomic repair and suggest that failure in overall repair is associated with cancer formation [45].

Wei et al. [47] found that repair in lymphocytes was reduced in patients with basal cell carcinoma (BCC) and Grossman [49] found an age-dependent decline in DNA repair that was associated with an increase in accumulated mutations in lymphocytes. Kovacs, Stucki, Weber, & Muller [50] found decreased DNA repair synthesis in the lymphocytes of women with invasive breast cancer and Pero et al., showed decreased repair synthesis in the mononuclear leukocytes of patients with adenomatous polyps [51] and colorectal cancer [52]. Further, Pero [52] found that those individuals that were genetically predisposed to colorectal cancer also had decreased rates of unscheduled DNA repair synthesis compared to healthy controls.

Psychological stress has been associated with altered repair of damaged DNA in both humans [53, 41, 54] and rats [55]. However, the direction of the change in repair seems

dependent on a number of factors such as the severity and chronicity of the stressor. For example, Kiecolt-Glaser et al.,[53] found that the lymphocytes drawn from high-distress psychiatric inpatients exhibited poorer repair than did the lymphocytes of low-distress patients. However, Forlenza et al., [41], and Cohen et al., [54] found increases in DNA repair in lymphocytes from healthy students during a stressful exam period. It is likely that the duration and intensity of distress in these later studies were less that in the inpatient sample studied by Kiecolt-Glaser et al., [53]. Both Forlenza et al., [41] and Cohen et al., [54] measured modest, transient stress in a sample of young, healthy, nonsmoking, community-dwelling students while Kiecolt-Glaser et al., [53] measured more severe distress in psychiatric inpatients, half of whom smoked. Another important difference concerns the timing of the putative stressor. Exam related stress could be considered an acute or episodic event of a relatively fixed duration. In contrast, mental illness severe enough to warrant hospitalization is typically more chronic in nature. The differential effects of acute and chronic stress exposure on DNA repair are unknown.

APOPTOSIS

Apoptosis is a highly organized process of programmed cell death. Initiation of apoptosis results in the discarding of unwanted (i.e. extra), damaged, and atypical (i.e. neoplastic) cells and can be induced by various physiologic and pathologic stimuli such as UV light, ionizing radiation, dietary carcinogens, and high levels of glucocorticoids [56]. Apoptosis is an active process that is structured and sequential and can be considered an important response to extensive DNA damage [57]. It requires energy for the transcription and translation of specific genes and can be distinguished from necrosis (pathologic death of cells).

Apoptosis is of vital importance in the protection against cancer. In response to extensive DNA damage, levels of the tumor suppressor protein p53 increase, arresting the cell cycle in G1

phase and allowing relevant DNA repair mechanisms to operate. If repair is unsuccessful, p53 levels continue to increase, inducing apoptosis by several mechanisms [58] Mutations in the p53 gene are exceedingly common and are reported to occur in 55% to 70% of human cancers [59] Loss of functional *p53* correlates with tumor aggressiveness and people with inherited defects in *p53* develop cancer at a high rate [60].

Many assay systems are available for measurement of apoptosis, including gel electrophoresis and ELISA [61], flow cytometry and laser scanning cytometry [62, 63], and various morphological and biochemical methods [64]. Measurement of apoptosis and related processes can be an important tool in biobehavioral studies of the impact of stress, adjustment, and other psychological variables in cancer progression.

Data examining the effects of stress on apoptosis are equivocal. Tomei, Kiecolt-Glaser, Kennedy, & Glaser [65] reported that psychological stress associated with medical student exam stress inhibited radiation-induced apoptosis in peripheral blood lymphocytes when treated with a tumor-promoting phorbol ester (12-o tetradeconoyl-phorbol-13-acetate (TPA). In contrast, Yin, Tuthill, Mufson, & Shi [66] and Tacic, Ovadia, Weiss, & Weidnefeld, [67] showed that chronic restraint stress in rats induced apoptosis in splenocytes and thymocytes, respectively. As with the data reported for DNA repair, discrepancies are likely explained by the methods and model systems used.

Psychosocial alteration of apoptosis could have significant impact on tumor progression through several mechanisms. If apoptosis of damaged cells is inhibited, an important mechanism protecting against neoplasia is lost. However, if apoptosis is induced or enhanced by psychological variables, there may be significant loss of immunosurveillance (due to loss of lymphocytes). Future work needs to clarify these important issues.

SOMATIC MUTATION

As previously discussed, when somatic cells receive DNA damage and this damage is unrepaired prior to cell replication, the damage becomes fixed in that cell's genome, leading to somatic mutation. What is important to remember here is that for somatic mutation to occur, there must be failures in multiple protective systems designed to prevent the survival of the mutation. Despite these elaborate and overlapping systems of protection, mutations do occur and the accumulation of mutations in somatic cells is thought to be the molecular basis for cancer [68].

For cells to become neoplastic, specific mutations must occur in specific genetic mechanisms. The first of these mechanisms concerns mutations in putative oncogenes. These genes involve regulation of cell growth and replication. Mutational activation of these genes most often leads to overexpression, unregulated expression, or inappropriate expression of the gene product causing an uncontrolled stimulation of cell growth [69]. The second genetic mechanism of mutation in cancer involves loss or inactivation of a series of genes termed tumor suppressor genes. Both copies of these suppressor genes must be inactivated for the cell to exhibit loss of tumor suppression. Because inactivation of tumor suppression involves loss of function mutations, the spectrum of events capable of inducing these mutations is much greater than those leading to activation of oncogenes. This makes loss of tumor suppression a more common event.

Since somatic mutation is the *de facto* final common pathway to tumor initiation and progression, understanding the impact of psychosocial variables on the accumulation of somatic mutation is of great importance. Although measurement of *in vivo* somatic mutation has become relatively commonplace in genetic toxicology and biodosimetry studies, quantitation of

background mutation frequency in biobehavioral oncology is lacking. There are currently several reliable assay systems capable of measuring somatic mutation in various hematopoietic tissues and each system has several advantages and disadvantages [69]. For example, the glycophorin A (GPA) assay is a rapid and relatively inexpensive procedure that measures mutation in RBC surface antigens resulting from *in vivo* allele loss at the GPA locus. The assay is performed using immunolabelling and flow cytometric procedures on a small sample of blood and provides data on a wide range of mutation mechanisms. A major limitation of this assay is that only 50% of the population has the appropriate blood type for the assay to be performed. In contrast, the hypoxanthine-guanine phosphoribosyltranferase (HPRT) assay is a lymphocyte-based assay that is applicable to the entire population. In addition to providing quantitative data regarding mutation, this assay allows for the molecular analysis of mutated cells. However, the HPRT assay is expensive and labor intensive, making it less suitable for larger, population-based studies.

Research suggests that the mutation frequency as determined by HPRT may vary more than 10-fold in a healthy, unexposed population [70]. While some of this variance may be accounted for by genetic differences in antioxidant scavenging systems and DNA repair systems, additional variance may be explained by the influence of psychosocial variables on these processes. Although several studies have examined the effects of behavioral lifestyle factors such as smoking and diet on mutation frequency [e.g. 71, 72], there are currently no studies evaluating the effects of psychological variables on somatic mutation. This may prove to be an important line of research if it could be demonstrated that variables such as stress directly influence *in vivo* mutational frequencies. These effects on mutation would need to be independent of their influence on health behaviors such as smoking, diet, or physical activity.

SUMMARY

This review focused on biobehavioral pathways linking psychosocial factors with cancer and suggested promising areas of investigation for behavioral researchers interested in understanding the role of psychosocial factors in cancer etiology and progression. Research shows that there is considerable variance in the functioning of each of these pathways and preliminary evidence suggests that psychological factors may explain some of this variance. Furthermore, research suggests that people with alterations in these processes (i.e. increased DNA damage, alterations in DNA repair, failures of apoptosis) are at increased risk for cancer.

However, the reviewed research demonstrates that the effects of psychosocial factors on the mechanisms of carcinogenesis are likely to be complex. Stressors vary with regard to their intensity and duration and it is likely that these variables will in turn moderate the impact of stress exposure on cancer processes. Moreover, it is likely that these carcinogenic processes interact with each other. For example, if stress increases oxidative stress, antioxidant systems should protect the DNA from damage. If antioxidant systems are suppressed or overwhelmed, the ensuing damage to DNA will initiate repair mechanisms. If DNA repairs fail, apoptosis in the damaged cell begins. If apoptosis is inhibited, somatic mutations accumulate leading to tumor initiation and promotion. This leaves immune surveillance which can be inhibited by chronic stress, as the last line of defense (see figure 1). In this way, small perturbations in one pathway will initiate a cascade of responses.

It is clear that much work remains to be done. Because of the inherent difficulties involved in studying the influence of psychosocial variables on clinical endpoints in cancer, research may progress by focusing on processes relevant to carcinogenesis. Furthermore, it will

be important to understand how various psychosocial variables interact with these processes simultaneously and how these interactions ultimately influence cancer risk.

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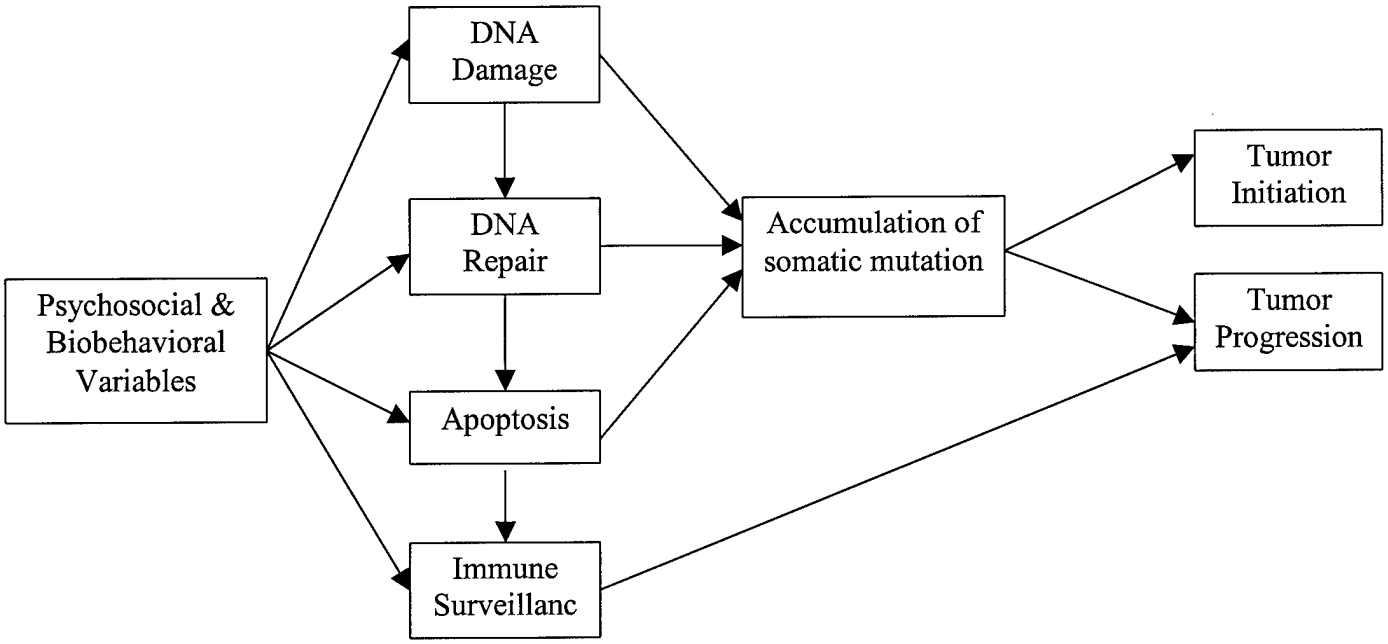
FIGURE 1: A Biobehavioral Model of Cancer Initiation and Progression


DNA Damage
DNA Repair
Apoptosis
Immune Surveillanc
Psychosocial & Biobehavioral Variables
Accumulation of somatic mutation
Tumor Initiation
Tumor Progression